Universidade de Aveiro

Departamento de Química

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Contribuição para a melhoria da qualidade nutricional do sorgo

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Bioquímica, realizada sob a orientação científica da Doutora Ivonne Delgadillo, Professora Associada com Agregação do Departamento de Química da Universidade de Aveiro e do Doutor António Barros, Investigador Auxiliar do Departamento de Química da Universidade de Aveiro

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Aos meus pais e ao meu marido

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palavras-chave Sorgo, proteínas, digestibilidade, valor nutricional, fermentação láctica.

resumo

Este trabalho teve como principal objectivo sugerir uma forma de processamento de sorgo adaptável à escala industrial que, sendo culturalmente aceite e sensível aos hábitos alimentares, aos factores sociais e às limitações económicas e tecnológicas das populações Africanas de consumo, pudesse dar origem a um produto alimentar seguro e enriquecido em termos nutricionais.

Numa primeira fase, efectuou-se um estudo comparativo entre os efeitos promovidos por diferentes formas de processamento: cozimento em água, aquecimento em banho-maria, pipocagem, germinação, fermentação e alta-pressão. Foram ainda determinadas as condições óptimas de aplicação do processo germinativo e da tecnologia de alta-pressão. Verificou-se que a fermentação, a germinação e a alta-pressão permitem uma melhoria significativa da digestibilidade proteica da farinha de sorgo. A pipocagem conduziu a uma redução da extractibilidade das proteínas não promovendo, contudo, alterações na sua digestibilidade. Comparativamente ao cozimento em água, que promove uma diminuição acentuada na digestibilidade proteica, o cozimento em banho-maria promove uma diminuição ténue e significativamente inferior. Deste estudo comparativo, foi possível concluir que a extractibilidade das proteínas não está correlacionada com a sua digestibilidade e que a água exerce um papel fundamental na diminuição da digestibilidade proteica com o aquecimento.

Numa segunda fase, pretendeu-se desenvolver um processo fermentativo que conduzisse a um produto de sorgo com características nutricionais incrementadas. Para tal, foram testadas daiferentes espécies de bactérias lácticas isoladas e conjugadas entre si. Foi ainda testada a adição de malte de sorgo previamente à fermentação e a adição de leveduras ao inóculo.

Com este estudo, foi possível concluir que a fermentação do sorgo com um inóculo constituído por culturas puras de *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus* permite a obtenção de uma preparação alimentar com características nutricionais melhoradas no que respeita ao balanço em aminoácidos essenciais, à digestibilidade da proteína e do amido e à viscosidade do produto final.

keywords Sorghum, proteins, digestibility, nutritional value, lactic fermentation.

abstract

This work had, as major purpose, to suggest a sorghum processing method adaptable to industrial scale which could be culturally accepted and sensitive to alimentary lifestyle, social factors and economic and technological limitations of the African populations, and could lead to a sorghum nutritional value improvement.

In the first stage of this work, it was carried out a comparative study of the effects promoted by different processing methods: boiling, heating in a water bath, popping, germination, fermentation and high-pressure. Optimal conditions for germination and high-pressure technology application were also studied. It was found that fermentation, germination and high-pressure allow a significant improvement in protein digestibility of sorghum flour. Although popping promoted a decrease on protein extractability, protein digestibility was not affected. Compared to boiling, which promotes a marked decrease on protein digestibility; cooking in a water bath promotes a tenuous and significantly lower decrease. This comparative study allows to conclude that protein extractability is not correlated with its digestibility and that water plays a fundamental role in the decrease of sorghum digestibility with thermal treatment.

In the second stage of this work, fermentation process was developed in order to obtain a sorghum product with improved nutritional quality. Single and mixed cultures of different lactic acid species were tested. The employment of sorghum malt prior to fermentation and the addition of yeasts to the inoculum were also tested.

With this study, it was concluded that fermentation of sorghum with an inoculum composed of *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus* leads to a sorghum fermented product with improved nutritional value as regards the essential amino acids balance, protein and starch digestibility and viscosity.

CAPÍTULO 1 – REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

O sorgo (*Sorghum bicolor* (L.) Moench) é o quarto cereal mais importante em termos de produção mundial. Constitui a base da dieta alimentar de mais de mil milhões de pessoas, em mais de 30 países, sendo seu consumo mundial superado apenas pelo arroz, trigo, milho e batata (Belton e Taylor, 2004, Board on Science and Technology for International Development, 1996). Mais de 35% da produção de sorgo é utilizada directamente para consumo humano, sendo a restante utilizada maioritariamente para alimentação animal, para produção de etanol e em produtos industriais (Awika e Rooney, 2004, FAO, 1995).

A sua utilização para alimentação humana assume especial importância nas regiões semi-áridas de África e da Ásia. Para as populações rurais mais carenciadas destas regiões, o sorgo constitui a principal fonte de energia, proteínas, vitaminas e minerais, sendo considerado "o alimento dos pobres" (Belton e Taylor, 2004). Este facto deve-se à escassez de outros alimentos, em parte devido às condições climáticas adversas ao crescimento de outras plantas. O facto de o sorgo ser uma planta extraordinariamente resistente a temperaturas elevadas e à escassez de água torna-o o principal meio de subsistência das populações que vivem quase exclusivamente da agricultura. Por outro lado, o consumo de sorgo apresenta também uma conotação religiosa. Os alimentos a base de sorgo, sobretudo os fermentados, são produzidos em casas, aldeias e pequenas indústrias, sendo posteriormente vendidos à população rural que os compra não apenas para alimentação, mas também para utilização em cerimónias sociais e religiosas (Iwuoha e Eke, 1996).

Nos países em desenvolvimento, em particular no continente Africano, o consumo de sorgo tem vindo a aumentar. Isto deve-se ao crescimento da população, mas também às políticas governamentais de incentivo ao seu processamento e à sua utilização industrial (Akintayo e Sedgo, 2001, Dicko *et al.*, 2006). O processo de urbanização conduziu a um aumento na procura por produtos tradicionais à base de sorgo, de tal modo que os processos tradicionais de preparação destes produtos já não conseguem satisfazer as necessidades dos consumidores. Como consequência, nalguns países Africanos estes processos tradicionais de preparação têm sido convertidos para a escala industrial de forma a fornecer produtos de valor acrescentado que possam suprir as necessidades das populações urbanas (Belton e Taylor, 2004, Gadaga *et al.*, 1999). Como tal, o consumo de alimentos à base de sorgo não se confina apenas às zonas rurais, mas também, e cada vez mais, às zonas urbanas.

De acordo com um estudo realizado aos hábitos alimentares em Polokwane (maior centro urbano da província de Limpopo, África do Sul), as razões pelas quais o sorgo retém um nicho da dieta urbana são (Bichard *et al.*, 2005):

- A disponibilidade de farinha de sorgo a preços razoáveis;

- A imagem do sorgo associada a alimento saudável - reforçada pela religião e pela medicina (aspecto que será abordado mais em pormenor juntamente com as características nutricionais do sorgo);

- O respeito das tradições Africanas - parte substancial da população deseja preservar a sua herança cultural e considera o consumo de sorgo como uma forma de regressar às raízes Africanas e à sabedoria dos seus ancestrais;

- Os hábitos herdados dos seus antepassados rurais – a maioria da população urbana cresceu em ambiente rural ou possui familiares em zonas rurais, tendo assim adquirido hábitos de consumo de sorgo.

- As questões religiosas – Muitas religiões incentivam ao consumo de sorgo ou utilizam-no em cerimónias religiosas. A maior Igreja Sul-Africana ("Zion Christian Church – ZCC") aconselha o seu consumo por questões de saúde e por respeito à tradição Africana.

O sorgo serve de matéria-prima para uma variedade enorme de pratos tradicionais, como por exemplo, pães, "cuscuz", panquecas, snacks, papas fermentadas e papas não fermentadas. É ainda o grão preferencial para a produção da cerveja Africana.

Devido à já referida resistência da planta de sorgo às condições adversas de temperatura e devido à sua versatilidade como matéria-prima alimentar, o sorgo é considerado por muitos como "o grão da África do século 21", apresentando um enorme potencial para ser o condutor do desenvolvimento económico da África (Board on Science and Technology for International Development, 1996, Taylor, 2003).

Muito embora um aumento drástico na produção e utilização mundial do sorgo seja um prognóstico praticamente certo (Board on Science and Technology for International Development, 1996), continuam a existir alguns impedimentos para que o sorgo possa alcançar o seu potencial internacional, como por exemplo:

- A má imagem associada ao sorgo. Em termos globais o sorgo continua a ser preterido pela ideia de se tratar de um "grão inferior", "cereal para alimentação animal" e "alimento das classes desfavorecidas", e

- O baixo valor nutricional. O sorgo apresenta problemas no que respeita à qualidade nutricional. As prolaminas do sorgo apresentam uma digestibilidade inferior comparativamente à de outros cereais. Por outro lado, a presença de taninos em certas variedades de sorgo representa um obstáculo à utilização de proteínas por parte do organismo humano. Isto torna-se especialmente preocupante no caso das populações que contam com o sorgo como principal fonte de nutrientes e energia, para as quais o sorgo não lhes confere o aporte proteico necessário.

Para que o sorgo possa ser a pedra-angular da segurança alimentar em África, é necessário o desenvolvimento de esforços no sentido de propor formas de processamento que confiram uma maior qualidade nutricional e que se ajustem à escala industrial de forma a satisfazer a procura por parte das populações de consumo de sorgo.

2. CLASSIFICAÇÃO E DESCRIÇÃO BOTÂNICA DA PLANTA

O sorgo pertence às Andropogonae e à família herbácea Poaceae. A cana-de-açúcar (*Saccharum officinarum*) que também pertence às Andropogonae, é parente próximo do sorgo. O género Sorghum é caracterizado por espigas que nascem aos pares. Trata-se de uma planta anual, embora seja uma erva perene e, nos trópicos, possa ser feita a sua colheita várias vezes ao ano (FAO, 1995).

Em 1753, Linnaeus descreveu no seu Species Plantarum três espécies de sorgo cultivado: *Holcus sorghum*, *Holcus saccaratus* e *Holcus bicolor*. Em 1794, Moench distinguiu o género *Sorghum* do género *Holcus*. Mais tarde, em 1805, Pearson propôs o nome de *Sorghum vulgare* para *Holcus sorghum* (L.). Em 1961 Clayton propôs o nome *Sorghum bicolor* (L.) Moench como o correcto para o sorgo cultivado, nome que se utiliza actualmente (FAO, 1995).

Harlan e de Wet (1972) publicaram uma classificação simplificada, na qual o sorgo cultivado é dividido em cinco grupos básicos: bicolor, guiné, caudatum, kafir e durra. O sorgo é vulgarmente conhecido na África ocidental por *milho grande* e *maíz da Guiné*, na África austral por *kafir*, no Sudão por durra, na África oriental por *mtama*, na Índia por *jowar* e na China por *kaoling* (Purseglove, 1972).

Tal como a maioria das linhagens angiospérmicas (plantas que dão flor), pensa-se que o sorgo tem aproximadamente 200 milhões de anos, tendo divergido de um ancestral comum ao milho, ao arroz e ao trigo (Paterson *et al.*, 2003).

O sorgo é uma planta do tipo C4, o que lhe confere a forma mais eficiente de realização da fotossíntese. Neste tipo de plantas, a fixação de CO_2 é feita pela PEP carboxilase, resultando num composto com 4 carbonos, o oxaloacetato, e seguidamente no malato. A utilização eficiente da luz do sol, também presente no milho e na cana-de-açúcar, é uma característica pouco comum entre as espécies de cultivo. Além disso, é tolerante às temperaturas elevadas e à escassez de água, o que lhe permite crescer em locais onde praticamente nenhuma outra planta consegue desenvolver-se consistentemente. Em situações de stress hídrico, a planta do sorgo apresenta mecanismos de conservação da humidade por redução da transpiração (por enrolamento das folhas), redução dos processos metabólicos e entrada em estado de quase latência até à chegada das chuvas (Board on Science and Technology for International Development, 1996).

A planta de sorgo é similar à do milho, sendo no entanto mais curta, normalmente com 75-127 cm de altura, e mais ampla. Apresenta um sistema de raízes fibrosas que permitem penetrar no solo até 2,5 m de profundidade. Do caule partem folhas em posições alternadas. A cabeça contém de 750 a 1250 sementes (FAO, 1995).

3. ESTRUTURA DO GRÃO DE SORGO

As características do grão de sorgo exibem uma grande diversidade no que diz respeito à cor, forma e tamanho, assim como noutros aspectos dos componentes anatómicos.

Tipicamente, o grão de sorgo apresenta 2-5 mm de comprimento e 2-3 mm de diâmetro na sua extremidade mais larga. A sua massa pode variar de 20 a 30 mg e a sua forma é normalmente esférica, com um embrião relativamente largo. A cor dos grãos varia amplamente, podendo ir de branco a tonalidades mais escuras como o castanho, sendo o vermelho e o amarelo cores muito comuns (Hoseney *et al.*, 1987).

A estrutura básica do grão é análoga à dos outros cereais, sendo os seus elementos anatómicos principais o pericarpo, o endosperma e o gérmen (Figura 1). A distribuição relativa destes três componentes anatómicos varia conforme a espécie de sorgo, sendo o peso médio do pericarpo 7,9%, do endosperma 82,3% e do gérmen 9,8% do grão (Hubbard *et al.*, 1950). A proporção entre o endosperma e o gérmen é geralmente de 8,4:1 (FAO, 1995).

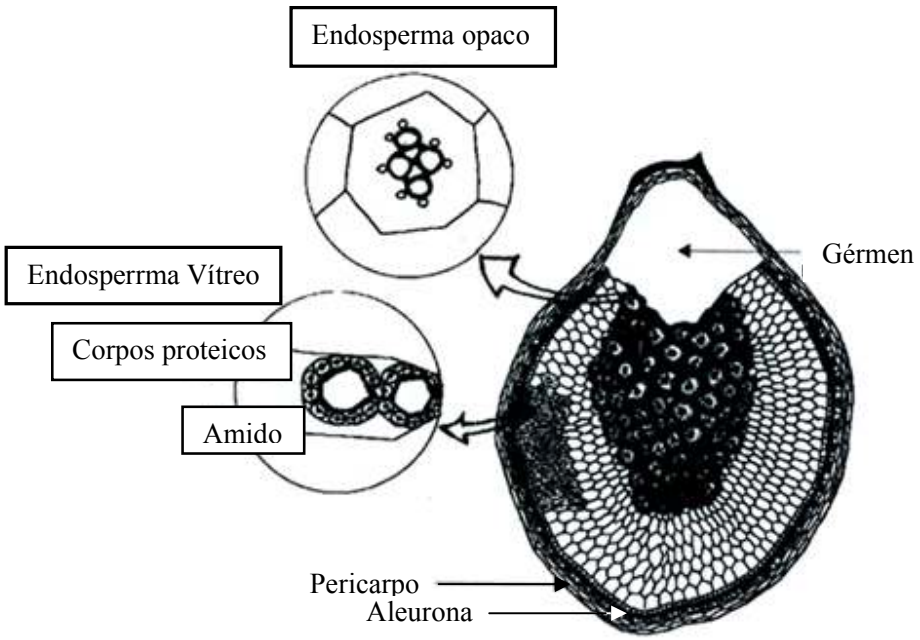

Figura 1: Estrutura do grão de Sorgo
(http://www.icrisat.org/text/research/grep/homepage/sgmm/chapter7.htm).

Pericarpo

O pericarpo, elemento mais externo do grão, é composto por três subcamadas: o epicarpo, o mesocarpo e o endocarpo (Serna-Saldivar e Rooney, 1995).

O epicarpo, subcamada mais externa do pericarpo, subdivide-se em epiderme e hipoderme. A epiderme é constituída por células grossas, alargadas e rectangulares com um revestimento cutínico na superfície exterior. Na epiderme encontram-se pigmentos. A hipoderme, por sua vez, é constituída por células ligeiramente menores e tem de uma a três camadas celulares de espessura (FAO, 1995).

O mesocarpo é a parte mais espessa do pericarpo, no entanto a sua espessura varia muito consoante o genótipo. O mesocarpo do sorgo, contrariamente ao dos outros cereais, contém pequenos grânulos de amido (1-4 μm). Certas linhagens de sorgo possuem um pericarpo muito fino com apenas alguns vestígios de células do mesocarpo (Hoseney *et al.*, 1987).

O endocarpo, subcamada mais interna do pericarpo, é composto por células transversais e por uma camada de células tubulares que transportam a humidade para o grão. Na maturação do grão, verifica-se o rompimento das camadas de células transversais e tubulares (FAO, 1995).

Certos genótipos de sorgo possuem uma camada pigmentada, denominada testa, onde se encontram os taninos condensados. O termo testa não é no entanto muito correcto, visto que todas as sementes maduras possuem testa mas nem todas apresentam essa camada pigmentada (Hoseney *et al.*, 1987). Essa testa pigmentada situa-se imediatamente abaixo do endocarpo e a sua cor e pigmentação são características genéticas. A espessura desta camada não é uniforme, verificando-se uma maior espessura na zona da coroa do grão, sendo a zona do embrião a mais delgada.

Endosperma

O maior componente do grão é o endosperma, importante tecido de armazenamento. A camada mais externa do endosperma é a aleurona, cujas células contêm corpos proteicos com pequenos corpos lipídicos e são ricas em minerais, vitamina B e algumas enzimas

hidrolizantes (FAO, 1995). As zonas periféricas do endosperma apresentam uma textura córnea.

As células do endosperma imediatamente abaixo das células de aleurona contêm grânulos de amido e proteínas. O amido contido nestas células, não está facilmente disponível ao ataque enzimático, a menos que as proteínas que lhe estão associadas também sejam atacadas (Chandrashekar e Kirleis, 1988). Uma grande parte dessas proteínas encontra-se em corpos proteicos. Esses corpos proteicos apresentam uma estrutura lamelar e têm 2-3 μm de diâmetro (Adams e Novellie, 1975). As proteínas que constituem os corpos proteicos são as prolaminas (Hoseney *et al.*, 1974, Sekinger e Wolf, 1973). A envolver os corpos proteicos e a manter os grânulos de amido inertes, encontra-se a matriz proteica. As proteínas que constituem essa matriz são as glutelinas (Sekinger e Wolf, 1973). Os corpos proteicos encontram-se em todas as células imaturas e, no caso do sorgo e de alguns cereais, persistem nas células maduras. No sorgo, o número de corpos proteicos diminui à medida que aumenta o seu conteúdo em amido, da zona periférica ao centro (FAO, 1995).

O endosperma do sorgo divide-se em duas partes visivelmente identificáveis. A parte mais externa é de aparência vítrea, enquanto a mais interna é opaca ou amilácea. A razão entre estas duas partes parece depender de questões genéticas. Os grânulos de amido no endosperma vítreo possuem forma poligonal e encontram-se empacotados sem espaços vazios entre eles. O número e tamanho dos corpos proteicos parecem estar relacionados com o teor em lisina da semente (Hoseney *et al.*, 1974, Sekinger e Wolf, 1973). No endosperma amiláceo há um número considerável de espaços vazios e os grânulos de amido são geralmente esféricos. A difracção de luz por parte destes espaços vazios no endosperma é responsável pela aparência opaca.

Gérmen

As duas partes principais do gérmen são o embrião e o escutelo. O escutelo é um tecido de armazenamento, rico em lípidos, proteínas, enzimas e minerais (FAO, 1995).

4. COMPOSIÇÃO QUÍMICA DO GRÃO DE SORGO

A composição química do sorgo é fortemente influenciada pelos factores genéticos e ambientais, sobretudo pelas práticas agronómicas, sendo o conteúdo proteico o mais variável.

O sorgo, à semelhança dos outros cereais, é predominantemente amiláceo e o seu conteúdo em proteína é comparável ao do milho, do trigo e do milho pequeno (FAO, 1995). O pericarpo do sorgo é pobre em proteína e cinzas, e rico em componentes fibrosos. O gérmen do sorgo é rico em cinzas, gorduras e proteína, porém muito pobre em amido (Serna-Saldivar e Rooney, 1995). Mais de 68% da matéria mineral total e de 75% dos lípidos do grão inteiro localizam-se no gérmen. O gérmen do sorgo é também rico em vitaminas B. O endosperma, porção maior do grão, é relativamente pobre em minerais e cinzas. Por outro lado, é um grande portador de outros componentes, contendo 80% das proteínas, 94% do amido e 50-75% das vitaminas B do grão inteiro.

O sorgo não possui vitamina A, à excepção de algumas variedades com endosperma amarelo que contêm pequenas quantidades de β-caroteno, precursor da vitamina A (FAO, 1995).

Hidratos de Carbono

O amido é o principal componente do grão de sorgo (seguido das proteínas, hidratos de carbono não amiláceos e gorduras) e a principal fonte de armazenamento de hidratos de carbono (Verbruggen, 1996, Verbruggen *et al.*, 1993).

O conteúdo em amido no sorgo varia de 56 a 73%, sendo o valor médio de 69,5% (Jambunathan e Subrahmanyan, 1988). Ocorre em grânulos de forma poligonal e esférica, com tamanhos que variam de 4 a 24 μm de diâmetro (Miller e Burns, 1970).

O conteúdo energético disponível num grão é determinado pela digestibilidade do amido, da qual depende a sua hidrólise por enzimas pancreáticas. O valor energético médio do sorgo é de 356 kcal/100g de farinha (Dicko *et al.*, 2006).

A digestibilidade do amido está fortemente relacionada com a textura do endosperma amiláceo, com o tamanho das partículas de farinha e com a sua natureza química, em

particular com o conteúdo de amilopectina e amilose. O teor de amilose no sorgo varia entre 23 e 30 % (Serna-Saldivar e Rooney, 1995). Hibberd *et al*. (1982) verificaram que no sorgo com baixo teor de amilose, a digestibilidade do amido é superior. As formas de processamento também podem ter efeito na digestibilidade do amido. Na preparação de alimentos à base de sorgo, métodos como o cozimento permitem que os grãos de amido se libertem da matriz proteica, tornando-os mais acessíveis ao ataque enzimático (Harbers, 1975). A presença de taninos no grão é outro factor que afecta a digestibilidade do amido, pois inibe a actividade enzimática (FAO, 1995).

Para além do amido, os grãos de sorgo contêm ainda açúcares solúveis, frutosanas, celulose, hemicelulose e substâncias pécticas.

Os principais açúcares solúveis redutores são a glucose e a frutose, com níveis que variam de 0,2 a 0,9% (Hoseney *et al.*, 1987). No grão não germinado pode encontrar-se ainda maltose.

Em níveis superiores, 0,3 a 2,1%, aparecem os açúcares solúveis não redutores (El Tinay *et al.*, 1979). O principal açúcar não redutor é a sacarose, que representa 75% dos açúcares solúveis. No grão imaturo existem ainda pequenas quantidades de oligossacarídeos, a maior parte dos quais constituídos por glucose e frutose, com preponderância da última.

Proteínas

O conteúdo em proteínas do sorgo (N x 6,25) é variável, podendo oscilar entre 7 e 14% (Hoseney *et al.*, 1987). Essa variação deve-se a factores genéticos e ambientais, exercendo também influência a data do cultivo, o tamanho da semente, a temperatura e, obviamente, o nível de fertilização com azoto (Burleson *et al.*, 1956, Waggle *et al.*, 1967).

Estudos realizados com duas espécies de sorgo de baixo teor em taninos, revelaram que aproximadamente 80% das proteínas se situam se no endosperma do grão, 16% no gérmen e apenas 3% no pericarpo (Taylor e Schussler, 1986).

Em 1914, Osborne e Mendel utilizaram um procedimento para a extracção sequencial de proteínas, que deu origem à classificação destas em função da sua solubilidade. Assim, as

proteínas do sorgo são agrupadas em albuminas (proteínas solúveis em água), globulinas (proteínas solúveis em solução salina), prolaminas (proteínas solúveis em solução alcoólica) e glutelinas (proteínas solúveis em solução alcalina).

As principais proteínas no grão do sorgo são as prolaminas (também denominadas kafirinas) e as glutelinas. As prolaminas representam aproximadamente 70% das proteínas totais e 80% das proteínas do endosperma (Hamaker *et al.*, 1995).

Em relação à localização das diferentes fracções proteicas, Chibber *et al.* (1978) verificaram que as albuminas e as globulinas estão localizadas nas partes mais externas do grão, as glutelinas estão uniformemente distribuídas no grão e as kafirinas encontram-se mais concentradas no interior do grão.

As proteínas de reserva de todas as sementes estão organizadas em corpos proteicos (Ashton, 1976). A composição dos corpos proteicos do sorgo foi determinada recorrendo a métodos de extracção, o que permitiu afirmar que são constituídos por kafirinas (Sekinger e Wolf, 1973, Taylor *et al.*, 1984a). Estudos realizados revelaram que as kafirinas são sintetizadas no retículo endoplasmático rugoso durante o desenvolvimento do endosperma, acumulando-se depois em corpos proteicos na forma de agregados interligados (Krishnan *et al.*, 1989, Taylor *et al.*, 1985b) A formação destes agregados é promovida pela maturação e secagem do grão (Oria *et al.*, 1995).

Em 1991, Shull *et al.* (1991) propuseram uma nomenclatura para as kafirinas (prolaminas do sorgo) baseada na já existente para as zeínas (prolaminas do milho). Esta nomenclatura tem como base as semelhanças dos pesos moleculares, solubilidade e estrutura destes dois tipos de prolaminas. De acordo com essa nomenclatura, as kafirinas dividem-se em α, β e γ. As kafirinas α são as constituídas por polipeptídeos de peso molecular 23 e 25 kDa e são solúveis em solução de *t*-butanol a 40-90% com 2-mercaptoetanol. As kafirinas β são constituídas por polipeptídeos de 16, 18 e 20 kDa e são solúveis em *t*-butanol a 10-60% com 2-mercaptoetanol. Por sua vez, as kafirinas γ são constituídas por um polipeptídeo de 28 kDa e são solúveis em *t*-butanol a 10-80% com 2-mercaptoetanol.

As diferenças na solubilidade destes três tipos de kafirinas podem explicar-se pelas suas composições em aminoácidos. As kafirinas α, que são extraídas apenas com elevadas concentrações de álcool, têm uma maior percentagem de aminoácidos hidrofóbicos como a fenilalanina, leucina e isoleucina. As kafirinas β apresentam uma menor percentagem destes aminoácidos, mas são relativamente ricas no aminoácido hidrofílico glicina. Desta característica resulta a sua extractibilidade com baixas concentrações de álcool. Por sua vez as kafirinas γ contêm também níveis elevados dos aminoácidos hidrofílicos glicina e histidina, resultando assim na sua extracção também a baixas concentrações de álcool. Tanto as kafirinas β com as γ apresentam ainda quantidades elevadas de cisteína, o que faz com que formem frequentemente ligações dissulfeto intermoleculares (Shull *et al.*, 1992). Shull *et al.* (1992) verificaram ainda, que as kafirinas α estão localizadas predominantemente no interior do corpo proteico, enquanto que as kafirinas β e γ, para além de se localizarem no interior, encontram-se também à superfície dos corpos proteicos.

Muitos estudos têm demonstrado que as kafirinas do sorgo podem ser divididas em duas fracções extractáveis: uma que pode ser extraída com solução alcoólica, denominadas kafirinas 1 ou kafirinas verdadeiras e outra, extraída do resíduo apenas com solução alcoólica e agente redutor, denominada glutelina solúvel em álcool (Jambunathan *et al.*, 1975, Sastry *et al.*, 1986), kafirina 2 (Mazhar e Chandrashekar, 1993) ou kafirina interligada (Guiragossian *et al.*, 1978, Oria *et al.*, 1995, Taylor *et al.*, 1984b).

El Nour *et al.* (1998) sugerem que essa diferença na solubilidade provém do facto das prolaminas extractáveis com agente redutor estarem presentes no endosperma na forma de polímeros de elevado peso molecular ligados por pontes dissulfeto, enquanto as extractáveis apenas com álcool estão na forma de cadeias polipeptídicas simples. A análise das proteínas por electroforese, feita pelos mesmos autores, revelou que em condições não redutoras observa-se, para além das bandas correspondentes às kafirinas α, β e γ, a presença de outras bandas correspondentes a polímeros de maior peso molecular. Ao efectuarem a mesma análise em condições redutoras, observaram que essas bandas de maior peso molecular desapareciam e que as outras se mantinham. Concluíram então que as kafirinas γ, que apresentam grandes quantidades de resíduos de cisteína, encontram-se ligadas às kafirinas α formando polímeros

de diferentes tamanhos e que estas duas proteínas apresentam a propriedade particular de formar pequenas estruturas ligadas por pontes dissulfeto que podem detectar-se por electroforese como entidades isoladas (extractáveis apenas com álcool), ou como parte do polímero principal (detectadas após extracção em banho de ultra-sons). As kafirinas β, por sua vez, não aparecem na forma de oligómeros ou polímeros, o que permitiu aos autores concluírem que estas proteínas actuam como uma ponte que mantêm ligados os oligómeros formados pelas kafirinas α e γ, tornando-os muito grandes para serem extraídos em condições não redutoras.

Vitaminas e Minerais

O sorgo é em geral uma importante fonte de vitamina B. Algumas variedades de sorgo de endosperma amarelo contêm β-caroteno, que pode ser convertido em vitamina A pelo corpo humano. No entanto, dada a natureza fotossensível dos carotenos e à variabilidade devida a factores ambientais, torna-se relativa a importância destas variedades de sorgo como fonte alimentar de vitamina A (FAO, 1995).

Tanner *et al.* (1947) verificaram que o sorgo, quando comparado com o milho, contém níveis similares de riboflavina, tiamina e piridoxina, níveis inferiores de caroteno e níveis superiores de ácido pantoténico, niacina, colina, ácido fólico e biotina. O sorgo, tal como se consome normalmente, não é uma fonte de vitamina C.

O sorgo é uma boa fonte para mais de 20 micronutrientes. É rico em fósforo, potássio, ferro e zinco (Anglani, 1998a). No entanto, segundo Kurien *et al.* (1960) mais de 70% do fósforo encontra-se na forma de fitato, que pode interferir na absorção de cálcio e outros minerais.

Polifenóis

Os polifenóis encontram-se amplamente distribuídos nas plantas. Nos grãos de sorgo com pericarpo castanho e testa pigmentada, verifica-se uma elevada concentração de polifenóis.

Os compostos fenólicos do sorgo podem classificar-se em ácidos fenólicos, flavonóides e taninos.

Os ácidos fenólicos, que podem encontrar-se livres ou esterificados, encontram-se nas camadas mais externas do grão e inibem o crescimento de microrganismos (FAO, 1995).

O sorgo é o único dos cereais capaz de produzir quantidades relativamente elevadas de taninos (Elkin *et al.*, 1996). Não contém taninos hidrolisáveis, mas certas espécies, resistentes aos pássaros, contêm taninos condensados. Os taninos têm a particularidade de proteger o grão do ataque dos pássaros e insectos e de prevenir a germinação pré-colheita. No entanto, as suas vantagens agronómicas vêm acompanhadas de desvantagens nutricionais e de uma diminuição na qualidade dos alimentos (Serna-Saldivar e Rooney, 1995). Estes aspectos serão aprofundados mais adiante, na abordagem aos aspectos nutricionais do sorgo.

Lípidos

Em geral, os lípidos existentes no sorgo são altamente insaturados, sendo uma grande percentagem (86%) apolar. Os triglicerídeos representam a maior porção de lípidos. Do total de ácidos gordos, 49% correspondem ao ácido linoleico, 31% ao oleico, 14% ao palmítico, 2,7 % ao linolenico e 2,1 % ao esteárico. Um elevado número de esteróis e mais de vinte tipos de ésteres foram também identificados no sorgo (Hoseney *et al.*, 1987).

Fibra Dietética

O termo fibra dietética descreve uma variedade de polissacarídeos vegetais, em particular a celulose, hemicelulose, pectinas, oligossacarídeos, gomas e vários compostos lenhificados, não hidrolisados pelas enzimas do tracto digestivo humano.

A principal fibra dietética no sorgo é a celulose. Encontra-se sobretudo no pericarpo e com uma variação de 1,2 a 5,2%. As hemiceluloses aparecem no sorgo como componentes da parede celular, ou como matriz que mantém as células unidas. O grão do sorgo contém de 2,5 a 5,6 % de pentosanas (Hoseney *et al.*, 1987).

5. ASPECTOS NUTRICIONAIS DO SORGO

5.1. Aspectos favoráveis que incentivam o consumo de sorgo

Os cereais em geral são componentes essenciais na dieta alimentar. Do ponto de vista nutricional, são fontes importantes de hidratos de carbono, proteínas, fibra dietética, vitaminas e compostos não-nutrientes (componentes alimentares que não são essenciais ao crescimento, mas que apresentam funções biológicas importantes). Os fitoquímicos, as fibras alimentares e os oligossacarídeos, presentes nas camadas exteriores dos grãos dos cereais, apresentam actividade preventiva para certas doenças (Katina *et al.*, 2005).

No caso do sorgo, existem vários compostos fitoquímicos (incluindo compostos fenólicos, esteróis vegetais e policosanóis), que são metabolitos secundários da planta ou constituintes celulares. Estes fitoquímicos possuem um impacto significativo na saúde humana, em parte devido à sua actividade anti-oxidante e às suas propriedades redutoras de colesterol (Awika e Rooney, 2004). Estudos realizados em porcos alimentados com uma dieta de 58% em sorgo (Klopfenstein *et al.*, 1981) revelaram uma redução nos níveis de colesterol. Estes efeitos foram superiores aos verificados com trigo, com a aveia e com o milho.

O sorgo contém níveis substanciais de uma gama variada de compostos fenólicos com propriedades benéficas para a saúde, em particular a actividade anti-oxidante, e possibilidade de utilização como nutracêuticos em alimentos (Dykes e Rooney, 2006). A presença de compostos fenólicos no sorgo, em particular de taninos, também está associada à redução da obesidade (Awika e Rooney, 2004). Este facto deve-se à ligação dos taninos às proteínas e aos hidratos de carbono, formando complexos insolúveis (inacessíveis ao ataque enzimático); ou às enzimas digestivas, inactivando-as.

O sorgo apresenta ainda níveis elevados de policosanóis, entre os quais o octacosanol (28:0) e o triacontanol (30:0). Estudos realizados por Hargrove *et al*., (2004) revelaram que misturas de álcoois C24-C34, incluindo o octacosanol e o triacontanol, reduzem os níveis de colesterol LDL (lipoproteína de baixa densidade) e aumentam os de HDL (lipoproteína de alta

densidade). Varady *et al.* (2003) concluíram ainda que os policosanóis são compostos promissores na prevenção e na terapia de doenças cardiovasculares.

O sorgo contém também amido resistente, o que lhe confere um menor índice glicémico e capacidade de aumento da saciedade, sendo assim aconselhável a diabéticos e obesos (Awika e Rooney, 2004, Dicko *et al.*, 2006). A presença de amido resistente como componente da fibra dietética do sorgo confere-lhe ainda outros benefícios à saúde, como por exemplo, prevenção do cancro do cólon, efeitos hipocolesterémicos, inibição da acumulação de gorduras, aumento da absorção de minerais e possibilidade de aplicação como probiótico (Sagilata *et al.*, 2006).

O sorgo não possui glúten sendo, portanto, um alimento recomendado a pessoas portadoras da doença celíaca e podendo ser utilizado na preparação de pães (Taylor *et al.*, 2006).

5.2. Aspectos desfavoráveis ao consumo de sorgo

Apesar das referidas vantagens nutricionais da utilização do sorgo como constituinte ou complemento da dieta alimentar, o sorgo apresenta características que tornam desvantajosa a sua utilização como componente principal da dieta humana. Tais características estão relacionadas com a fraca qualidade nutricional das proteínas, aspecto que contribui para o elevado grau de subnutrição das populações carenciadas da África e Ásia para as quais o sorgo representa o principal aporte nutricional.

O que define a qualidade nutricional de uma proteína é a sua composição em aminoácidos essenciais, digestibilidade e biodisponibilidade (Finley, 1989).

Os aminoácidos essenciais são aqueles que, tanto na criança como no adulto, vão ser utilizados pelo organismo directamente em reacções e processos metabólicos específicos, de onde provém a necessidade da sua ingestão. São aminoácidos essenciais a fenilalanina, isoleucina, leucina, lisina, metionina, treonina, triptofano e valina. Há ainda os aminoácidos semi-essenciais, que são os sintetizados a partir dos essenciais. São eles a tirosina, arginina, histidina e cisteína (Romo e Linkswiler, 1969).

O sorgo apresenta uma composição em aminoácidos semelhante à dos restantes cereais, em que a lisina aparece como aminoácido limitante (Wall e Blessin, 1970). De forma a incrementar o valor proteico do sorgo, numerosos estudos têm sido efectuados na tentativa de encontrar variedades de sorgo ricas em lisina, por exemplo através de modificações genéticas (Serna-Saldivar e Rooney, 1995).

Com base no padrão estabelecido para humanos pela Organização Mundial de Saúde (OMS) (1973) verifica-se que, além da lisina, o sorgo apresenta ainda a metionina, cisteína e isoleucina como aminoácidos limitantes (Neucere e Sumrell, 1979).

A composição em aminoácidos do endosperma do grão de sorgo segue o mesmo padrão do grão inteiro, sendo mais abundante em ácido glutâmico, leucina, prolina e alanina e deficiente em lisina. No gérmen e no pericarpo, por sua vez, predominam a glicina, a arginina e a lisina, sendo o conteúdo de lisina duas vezes superior ao do endosperma e do grão inteiro. À excepção da isoleucina, a composição em aminoácidos das proteínas do gérmen está em conformidade com o padrão estabelecido para proteínas de elevada qualidade (Taylor e Schussler, 1986). No entanto, este aspecto torna-se irrelevante para a avaliação da qualidade das proteínas do sorgo, uma vez que o gérmen e o pericarpo são as partes do grão normalmente removidas durante o processamento.

Estudos sobre a composição em aminoácidos das diferentes fracções proteicas revelaram que as albuminas e globulinas e são bem equilibradas no que diz respeito à sua composição em aminoácidos essenciais, com quantidades elevadas de lisina e triptofano. As glutelinas são ricas em aminoácidos essenciais e ácido glutâmico, e contêm níveis moderados de leucina. As prolaminas, por sua vez, contêm quantidades elevadas de ácido glutâmico, prolina e leucina, mas são bastante pobres em lisina, arginina, histidina e triptofano (Ahuja *et al.*, 1970). Por essa razão, o baixo conteúdo em lisina é por vezes atribuído ao elevado teor em prolaminas no sorgo (Neucere e Sumrell, 1979).

À semelhança do que ocorre com outros cereais, o sorgo apresenta ainda um teor desproporcionado de leucina. De acordo com Deosthale e Mohan (1970), para que uma fracção proteica possa ser considerada nutricionalmente segura como fonte de proteínas, deve

apresentar uma razão leucina/lisina inferior a 4,6. Estudos feitos por Neucere e Sumrell (1979) revelaram que, apesar das albuminas, globulinas e glutelinas apresentarem uma relação leucina/lisina inferior a 4,0, o mesmo não se verifica com as prolaminas, fracção mais abundante.

O aspecto mais negativo na qualidade das proteínas do sorgo está, no entanto, relacionado com a sua digestibilidade. As proteínas do sorgo são as únicas entre as proteínas de plantas comestíveis, cuja digestibilidade diminui marcadamente com o cozimento (Axtell *et al.*, 1981, Eggum *et al.*, 1983, Mitaru *et al.*, 1985, Oria *et al.*, 1995). Esta é, aliás, a principal razão pela qual o sorgo é preterido aos outros cereais em termos alimentares. A diminuição da digestibilidade proteica com o cozimento tem vindo a ser alvo de inúmeros estudos no sentido de perceber as principais causas envolvidas e de propôr formas de as reverter.

Em 1960, Kurien *et al.* (1960) desenvolveram estudos em crianças com idades compreendidas entre 10 e 11 anos de idade e concluíram que, quando alimentadas com arroz, os valores da digestibilidade aparente das proteínas era de 75 %, enquanto quando alimentados com sorgo, os valores desciam para 55 %. Estes resultados foram confirmados por Daniel *et al.* (1966) em estudos com jovens alimentadas com dietas a base de sorgo. Em 1981, MacLean *et al.* (1981) publicaram resultados de estudos de balanço de Azoto efectuados em crianças peruanas, que mostram que a digestibilidade do sorgo cozido é significativamente menor que a do trigo, milho e arroz cozidos (46 % vs. 81 %, 73 % e 66 %, respectivamente).

Devido às restrições associadas ao desenvolvimento de ensaios *in vivo,* Axtell *et al.* (1981) estudaram as mesmas variedades de sorgo utilizadas por MacLean, mas recorrendo a ensaios *in vitro* utilizando pepsina. Os resultados obtidos por Axtell *et al.* foram similares aos obtidos com humanos, verificando-se, no entanto, que os valores de digestibilidade diminuíam consideravelmente após o cozimento. Estes resultados deram a indicação que esta forma de processamento do sorgo altera as suas proteínas tornando-as menos digeríveis.

Estudos *in vivo*, desta vez realizados com animais, confirmaram a redução da digestibilidade das proteínas do sorgo com o cozimento. Eggum *et al*. (1983) utilizando ratos, verificaram que nas proteínas do sorgo cozido a digestibilidade era 7 % menor que nas proteínas do sorgo cru.

Em ensaios com galinhas, a diminuição verificada na digestibilidade das proteínas do sorgo cozido em relação a do sorgo cru era de 31,5 % (Mitaru *et al.*, 1985).

Hamaker *et al.* (1986) efectuaram novos ensaios *in vitro* que concluíram que com pepsina, tripsina/quimotripsina ou ambos os sistemas enzimáticos, a digestibilidade do sorgo diminuía com o cozimento enquanto a do milho se mantinha. Estes resultados foram confirmados por Agudelo *et al.* (1998). Hamaker *et al.* demonstraram ainda, recorrendo a electroforese em gel, que as kafirinas são a fracção proteica responsável pela diminuição da digestibilidade das proteínas.

O enorme esforço desenvolvido, desde há algumas décadas, no sentido de se chegar à causa da reduzida digestibilidade do sorgo, sobretudo com o cozimento, resultou em inúmeras publicações que apontam para diferentes factores. Algumas dessas publicações por vezes são ambíguas e continua-se, no entanto, sem saber qual a causa predominante, ou até mesmo se será o somatório dos diversos factores que estará por trás da reduzida digestibilidade do sorgo face aos outros cereais. De entre os factores apontados, estão:

- A formação de pontes dissulfeto entre as proteínas (Agudelo *et al.*, 1998, Axtell *et al.*, 1981, Duodu *et al.*, 2001, Hamaker *et al.*, 1987, Hamaker *et al.*, 1986, Hamaker, 1994, Oria *et al.*, 1995, Rom *et al.*, 1992). Este pressuposto é suportado pelo facto do sorgo conter níveis de ligações dissulfeto superiores às do milho e da digestibilidade assumir valores comparáveis ao do sorgo cru quando utilizados agentes redutores durante o cozimento (Agudelo*, et al.*, 1998). Os estudos desenvolvidos por Hamaker (1994) e Oria *et al.*(1995) permitiram concluir, ainda, que as kafirinas α são digeridas mais lentamente devido à sua localização interna e que as kafirinas β e γ são as primeiras a serem degradadas por se situarem na periferia dos corpos proteicos. Com o cozimento, a formação de ligações dissulfeto entre as kafirinas β e γ torna-as mais resistentes ao ataque enzimático, dificultando assim ainda mais o acesso das proteases às kafirinas α.

- A própria estrutura dos corpos proteicos do grão: Em espécies de sorgo altamente digerível, verificou-se que os seus corpos proteicos apresentavam uma forma irregular e marcadamente diferente das outras espécies, com invaginações profundas ou pregas

que, por vezes, alcançavam a parte central dos corpos proteicos formando lóbulos de forma irregular. Verificaram, ainda, que nestes corpos proteicos não estavam presentes as zonas escuras periféricas que aparecem nas espécies normais, e que apareciam inclusões escuras na base das pregas. (Oria *et al.*, 2000, Weaver *et al.*, 1998). Com base nas evidências que sugerem que é a localização periférica das kafirinas γ nos corpos proteicos que impedem a digestão das outras kafirinas, estes autores concluíram que a alteração da estrutura dos corpos proteicos e a mudança da localização das kafirinas γ para inclusões na base das pregas estão directamente relacionadas com a digestibilidade proteica superior destas espécies invulgares de sorgo.

- A presença de taninos (Agudelo *et al.*, 1997, Babiker e El-Tinay, 1992, Chavan *et al.*, 1979, Price e Butler, 1978, Price *et al.*, 1980, Tamir e Alumot, 1969). Os taninos combinam-se com proteínas exógenas e endógenas, inclusive com as enzimas do tracto digestivo, afectando assim a utilização dessas proteínas (Eggum e Christensen, 1975).

- Outros factores ainda em estudo, como a formação de complexos físicos ou químicos entre o amido e as proteínas, entre amido e lípidos (Englyst *et al.*, 1983) e entre proteínas e lípidos (Nunes, 2004); e a existência de proteínas de 45 e 47 kDa com o cozimento (Nunes, 2000). De acordo com Nunes estas proteínas, resistentes à digestão com pepsina e às condições redutoras, podem estar relacionadas com a diminuição da digestibilidade com o cozimento. O facto de estas proteínas não serem reduzidas põe em causa a formação de pontes dissulfeto como causa da reduzida digestibilidade do sorgo cozido.

A baixa densidade energética e nutriente é outro aspecto nutricional importante a considerar, sobretudo quando o sorgo é utilizado como alimento complementar ao leite materno.

O primeiro ano de vida, que normalmente coincide com a introdução de alimentos complementares, é normalmente o mais vulnerável à ocorrência de subnutrição nos países Africanos (Mosha e Vicent, 2004). A partir dos seis meses de idade, os requerimentos nutricionais passam a ser superiores aos fornecidos pelo leite materno. Durante este estágio do

desenvolvimento, o metabolismo das crianças requer quantidades maiores de energia e proteínas, sendo necessários suplementos alimentares de modo a satisfazer os requerimentos nutricionais (Lalude e Fashakin, 2006). Contudo, os alimentos complementares à base de leite (utilizados nos países desenvolvidos) são muito caros em África, não estando acessíveis à maioria da população. Por esta razão, utilizam-se normalmente papas de cereais, que consistem em suspensões aquosas de milho, arroz ou sorgo (Onilude *et al.*, 1999).

As papas de sorgo, no entanto, têm que ser diluídas para uma concentração de 5 a 10 % de modo a apresentarem consistência adequada para serem ingeridas por crianças nestas idades. Ao diluir, obtêm-se grandes volumes de papas com densidade energética e nutriente insuficientes para fornecer os requerimentos mínimos necessários (Belton e Taylor, 2004, Bond *et al.*, 2005, Onyango *et al.*, 2004, Sanni *et al.*, 2001, Sanni *et al.*, 1999a). A capacidade gástrica das crianças, (aproximadamente 30-40mL/kg de peso corporal), e a frequência diária de consumo (2 a 3 refeições por dia), impedem a ingestão de grandes volumes de alimento por dia (Traoré *et al.*, 2004). Uma vez que os volumes ingeridos não fornecem as quantidades adequadas de proteínas e energia, a deficiência de nutrientes começa a ser um problema sério na fase de transição entre o leite materno e a dieta adulta.

Nos países Africanos, assim como noutros países subdesenvolvidos, a alimentação infantil tem sido alvo de grande preocupação por parte da comunidade científica e de organizações governamentais e não governamentais, devido ao elevado grau de subnutrição verificado nas crianças. Ao défice nutricional, estão associadas elevadas taxas de mortalidade, doenças e retardamento do crescimento físico e do desenvolvimento mental (Onilude *et al.*, 1999). Nesses países, a subnutrição é responsável por 50% da mortalidade infantil (Thaoge *et al.*, 2003).

6. UTILIZAÇÃO MUNDIAL DO SORGO

O sorgo é dos cereais mais tolerantes à escassez de água, com requerimentos nutricionais mínimos durante o seu crescimento. Com o aumento da população mundial e concomitante diminuição dos recursos de água, o sorgo poderá, no futuro, representar uma alternativa viável

em termos alimentares (Taylor *et al.*, 2006). Enquanto nos países de África e da Ásia o sorgo é um alimento indispensável a milhões de pessoas, na maioria dos países desenvolvidos constitui um recurso subutilizado. Nos Estados Unidos, na Austrália e noutros países desenvolvidos o sorgo é cultivado apenas para consumo animal (Dicko *et al.*, 2006).

6.1. Distribuição geográfica e produção mundial de sorgo

O sorgo cultivado actualmente tem origem no progenitor selvagem que pertence à subespécie *verticilliflorum*. Pensa-se que a primeira espécie de sorgo cultivado surgiu há cerca de cinco mil anos na Etiópia (Mann *et al.*, 1985).

O sorgo foi levado da África Oriental para a Índia, provavelmente no primeiro milénio antes de Cristo. O seu tráfico em embarcações, entre a África e a Índia, ocorreu durante cerca de três mil anos. A sua difusão para o sudeste asiático e para a China parece ter-se verificado no início da era cristã ou, no caso da China, ainda antes através das rotas do comércio da seda.

Pensa-se que o sorgo migrou da África ocidental para a América Latina, como maíz da Guiné, em meados do século XIX com os comerciantes de escravos que faziam a rota Europa - África - América Latina. No entanto, o seu cultivo só adquiriu importância no século XX.

A produção mundial anual de sorgo é de 65,53 milhões de toneladas, apresentando uma área cultivada de 46 milhões de hectares. A produção mundial anual, no entanto, tem vindo a aumentar com o aperfeiçoamento das técnicas de cultivo e com a introdução de variedades seleccionadas. Os principais países produtores de sorgo são os Estados Unidos, a Nigéria, a Índia, o México, o Sudão, a China, a Argentina, a Etiópia, o Brasil, a Austrália e o Burquina Faso. O líder mundial de produção e consumo de sorgo por habitante é o Burquina Faso e, em área cultivada, é a Índia (FAO, 2005).

Na maioria dos países do Oeste Africano, o sorgo ocupa 50% da área total de cultivo de cereais. O rendimento de produção nestes países é, no entanto, de apenas 1000 a 3000 kg/ha, em contraste com os 3000 a 4000 kg/ha verificados na Argentina, na China e nos Estados Unidos (Dicko *et al.*, 2006). O baixo rendimento de produção verificado nos países Africanos deve-se essencialmente ao stress biótico (insectos, fungos, doenças, ervas daninhas, etc.) e

abiótico (secas, qualidade do solo, foto-período, etc.) (Dicko *et al.*, 2006). De acordo com Belton e Taylor (2004), a produção mundial de sorgo está equiparada à do milho sendo, contudo, a sua área cultivada aproximadamente o dobro da do milho. Segundo os mesmos autores, apesar da produção mundial de sorgo ter vindo a aumentar nos últimos anos, este aumento encontra-se dependente do cultivo de áreas cada vez maiores. O aumento contínuo das áreas de cultivo é ambientalmente prejudicial e insustentável a longo prazo, razão pela qual têm vindo a ser desenvolvidos esforços no sentido de incrementar a agricultura em África. Exemplo disso é a agricultura intensiva praticada na África do Sul, com variedades seleccionadas ou híbridos, resultando em rendimentos maiores e comparáveis aos do milho. Maiores rendimentos de produção de sorgo são essenciais não apenas para a sustentabilidade da agricultura, mas também para um aumento da comercialização do sorgo (Belton e Taylor, 2004)

6.2. Principais alimentos a base de sorgo

Em África e na Ásia, existe uma variedade de produtos à base de sorgo que incluem pipocas, sêmolas, produtos cozidos a vapor, papas, pães, panquecas, pastas, snacks, bebidas não alcoólicas e cervejas opacas. As papas e os pães podem ser fermentados ou não. Uma característica importante de muitos destes produtos é o recurso à fermentação, à germinação ou a ambos durante a sua preparação. Estas tecnologias simples permitem incrementar as propriedades funcionais e nutricionais dos alimentos à base de sorgo.

Os alimentos podem ainda ser feitos à base dos grãos inteiros cozidos ou de farinha de sorgo obtida por moagem tradicional ou industrial dos grãos.

Na Índia, os grãos de sorgo podem ser tostados inteiros, fazendo-se rebentar em pratos especiais ou em banhos de areia aquecidos ao fogo, obtendo-se assim pipocas de sorgo (Subrahmanyan e Jambunathan, 1980). Os grãos inteiros descascados podem ainda ser cozidos em água e servidos como arroz (Subramanian *et al.*, 1982). Na África ocidental, a sêmola de sorgo é cozida a vapor para produzir um alimento espesso e uniformemente gelatinizado denominado cuscuz (FAO, 1995).

O alimento mais simples e comum que se pode preparar à base da farinha de sorgo são as papas. As papas preparam-se adicionando, aos poucos e com agitação vigorosa, farinha à água fervente até que se obtenha a consistência desejada. As papas podem ser mais ou menos consistentes, dependendo da concentração de farinha empregue. Normalmente, as papas mais consistentes são sólidas e comem-se com as mãos, acompanhadas de vegetais, carne, peixe, azeite e/ou especiarias. As papas menos consistentes são líquidas e consomem-se bebendo ou comendo com auxílio de utensílios. São consumidas no desjejum ou por mães em fase de amamentação e por crianças lactentes (Murty e Kumar, 1995).

Exemplos de papas de maior consistência são o *tô* (Nigéria, Mali e Burquina Faso), o *ugali* (Quénia, Tanzânia e Uganda), *sankati* (Índia), o *tuwo* (Nigéria), *aceda* (Sudão), *bogobe* (Botsuana) e *sadza* (Zimbabué). O *tô* pode ser ácido, e para tal adiciona-se extracto de tamarindo ou sumo de limão; ou alcalino, por adição de cinzas de madeira. Como exemplos de papas de menor consistência há o *ugi* (Quénia, Tanzânia), *edi* (Uganda) *obushera* (Uganda), *kunu* (Nigéria) e *ambali* (Índia) (FAO, 1995, Murty e Kumar, 1995). O *uji* pode ser uma papa fermentada ou não, e o *bushera* é uma papa preparada a partir de grãos germinados.

As papas de menor consistência, como referido anteriormente, são muitas vezes utilizadas como alimento complementar ao leite materno durante o desmame. A preparação das papas para lactentes é por vezes feita por adição de sorgo germinado. O malte de sorgo é adicionado às papas espessas, liquefazendo-as pela acção de alfa-amilases que clivam o amido em dextrinas e maltose. Obtêm-se assim papas com viscosidades inferiores a 3000 cP (consistência adequada para alimentação de crianças com menos de 3 anos), mantendo-se a densidade nutriente. O *togwa* é um exemplo de papa utilizada na alimentação de lactentes em que, para além da utilização do sorgo maltado, se recorre também à fermentação (Belton e Taylor, 2004).

Na África e na Ásia, as papas fermentadas são mais populares do que as não fermentadas. Nestes países é comum a preparação de diversos produtos fermentados que, para além das papas, incluem ainda pães, bebidas alcoólicas e não alcoólicas. Na tabela 1 estão apresentados

alguns alimentos tradicionais fermentados a base de sorgo. Os produtos fermentados mais comuns à base de sorgo são o *ogi*, o *bogobe*, o *injera*, o *kisra* e o *burukutu*.

O *ogi* é das papas mais populares, sendo consumida não apenas por adultos, mas também por crianças como alimento complementar. Apresenta uma textura suave, um sabor ácido e um aroma característico. Na sua preparação tradicional, os grãos são embebidos em água durante 1 a 3 dias e, seguidamente, são moídos e peneirados para remoção do farelo, casca e gérmen. Após 2 a 3 dias de fermentação, obtém-se então o *ogi*, papa de aspecto esbranquiçado e amiláceo (Iwuoha e Eke, 1996).

O *bogobe* é uma papa de maior consistência que o *ogi*, preparada no Botsuana. Na sua preparação, os grãos de sorgo são lavados, descascados e misturados com água até a formação de uma pasta que é levada a fermentar em recipiente fechado por 24 horas. A mistura resultante é, finalmente, cozida durante 15 minutos (FAO, 1995).

Os pães feitos a base de sorgo apresentam um volume inferior e uma massa mais elástica, seca e escura que os preparados com farinha de trigo. O *injera* é o pão de sorgo mais popular da Etiópia. É uma espécie de panqueca de 50 cm de diâmetro com textura do tipo das favas de mel. Os grãos de sorgo são descascados manual ou mecanicamente e moídos numa farinha que é então utilizada na preparação do *injera*. À farinha é adicionada água numa proporção de 4:1 (m/v) de modo a formar-se uma massa. A seguir, é adicionada uma papa proveniente de uma fermentação anterior que funciona como inóculo. A mistura é amassada e adiciona-se novamente água. Após fermentação por 48 horas, a massa é finalmente cozida para dar origem ao pão (FAO, 1995). O *injera* é o único pão que, apesar de não possuir glúten, é levedado. Cozendo parte da farinha de sorgo para promover a gelatinização do amido, o dióxido de carbono produzido na fermentação fica retido na massa sendo libertado no momento da cozedura do pão (Belton e Taylor, 2004).

O *kisra* é outro pão fino, do tipo panqueca, muito consumido no Sudão. Apresenta um sabor bastante ácido e é preparado por mistura da farinha em água até a obtenção de uma pasta fina que se deixa a fermentar de 12 a 24 horas. Após o período de fermentação, adiciona-se água até à obtenção da consistência de um batido e coze-se (FAO, 1995).

O *khamir* é também um pão, produzido na Arábia Saudita por mistura de farinha de sorgo, água e algumas especiarias e fermentação por 24 horas a 30ºC. Em seguida, duas outras fermentações são realizadas utilizando o inóculo da fermentação anterior (Gassem, 1999).

Os pães de sorgo também podem ser pães não fermentados, como por exemplo o *roti* e *chapatti* na Índia e o *tuwo* nalgumas partes da Nigéria (FAO, 1995).

A utilização de sorgo para preparação de bebidas tradicionais fermentadas é bastante popular em vários países Africanos. Embora as bebidas não constituam grandes alimentos, são usadas como fonte de energia em vários países. A Cerveja Sul-Africana distingue-se das outras cervejas por ser mais ácida e menos carbonada. O processo de fermentação não é interrompido durante a produção, mantendo-se até a altura do consumo. A cerveja é consumida numa forma não refinada, incluindo substratos não fermentados e microrganismos (Mwesigye e Okurut, 1995). O *burukutu* é a cerveja de sorgo mais popular, consumida na Nigéria, Guiné e Benin. Na sua preparação, após os grãos serem embebidos em água por 1 dia, a água é drenada e os grãos são germinados por 2 dias. Posteriormente, os grãos são secos ao sol e moídos para obtenção de uma farinha. A farinha é então misturada com água e fervida por 3 a 4 horas até se obter uma papa. A papa é decantada e após nova adição de água é novamente fervida por 3 horas. A mistura é mantida a temperatura ambiente durante 24 horas e repete-se o procedimento de adição de água e fervura. Com o arrefecimento da mistura, obtém-se um sobrenadante e um sedimento que é fermentado durante 1 dia para obtenção do *burukutu* (Iwuoha e Eke, 1996).

O sorgo é ainda consumido na forma de pastas, como o esparguete, embora seja menos indicado que o trigo por não conter glúten e por ter uma temperatura de gelatinização superior (FAO, 1995). A farinha de sorgo também é utilizada em biscoitos e snacks (Anglani, 1998b).

As variedades de sorgo brancas, utilizadas para preparação de snacks expandidos, biscoitos e produtos étnicos, têm vindo a ganhar popularidade no Japão (Awika e Rooney, 2004). Estas variedades também têm vindo ser utilizadas nos Estados Unidos, como substitutas do trigo na preparação de pães para pessoas alérgicas ao glúten (Fenster, 2003). As variedades de sorgo com taninos (variedades castanhas), por sua vez, são preferidas por algumas culturas Africanas

por se considerar que as papas preparadas com este tipo de sorgo "permanecerem mais tempo no estômago", permitindo que os trabalhadores rurais se mantenham saciados durante a maior parte do dia de trabalho nos campos (Awika e Rooney, 2004). Por outro lado, e sobretudo nas mulheres Africanas, existe ainda a ideia de que o sorgo castanho está associado a alimento de maior valor nutricional, mais saudável (Bichard *et al.*, 2005).

O facto de a fermentação conferir vantagens nutricionais e de segurança alimentar está relacionado com a sua extensa aplicação na preparação dos alimentos tradicionais a base de sorgo. Nestas fermentações, estão envolvidas misturas de microrganismos, compreendendo maioritariamente bactérias lácticas heterofermentativas mesofílicas que, ao promoverem modificações estruturais nas proteínas, promovem um aumento na sua digestibilidade. Por outro lado, a diminuição no pH dos alimentos, provocada por estes microrganismos, confere-lhes uma protecção contra o crescimento de bactérias patogénicas. Este aspecto é de extrema importância em África, onde muitas pessoas continuam a não ter acesso a água microbiologicamente segura (Belton e Taylor, 2004).

Para além das referidas vantagens conferidas pelas bactérias lácticas presentes nos produtos fermentados de sorgo, a utilização destes microrganismos e dos seus produtos de fermentação em alimentos probióticos começa a ser uma nova tendência. Um exemplo disso é o *Dogik,* alimento derivado do já descrito *ogi*. O Dogik foi desenvolvido utilizando bactérias lácticas como culturas propagadoras ("starter") que apresentam actividade anti-microbiana contra algumas doenças que provocam diarreias (Blandino *et al.*, 2003).

Tabela 1: Principais alimentos e bebidas de sorgo fermentado (Anglani, 1998b, Blandino *et al.*, 2003, FAO, 1995, Murty e Kumar, 1995).

Nome	***Tipo de Alimento***	***Microrganismos Encontrados***	***Região de Consumo***
Bogobe	Papa líquida	Não identificados	Botsuana
Burukutu	Bebida alcoólica de sabor avinagrado	*Saccharomyces cerevisiae, S. chavelieri, Leuconostoc mesenteroides, Candida, Acetobacter*	Nigéria, benin, e Gana
Injera	Panqueca	*Candida guilliermondii*	Etiópia
Kisra	Panqueca	Não identificados	Sudão
Mahewu	Bebida não-alcoólica ou papa espessa	*Lactococcus lactis, Streptococcus lactis*	África do Sul
Merissa	Bebida alcoólica	*Saccharomyces*	Sudão
Nasha	Papa líquida	*Streptococcus, Lactobacillus, Candida, S.cerevisiae*	Sudão
Ogi	Papa líquida	*Lactobacillus plantarum, S. cerevisiae, Candida mycoderma, Corynebacterium, Aerobacter, Rhodotorula, Cephalosporium, Fusarium, Aspergillus and Penicillium*	Nigéria e Oeste Africano
Otika	Bebida alcoólica	Não identificados	Nigéria
Pito	Bebida Alcoólica	*Geotrichum candidum, Lactobacillus, Candida*	Nigéria e Gana
Cerveja Sul Africana	Bebida Alcoólica	Bactérias lácticas e leveduras	África do Sul
Talla	Bebida Alcoólica	Não identificados	Etiópia
Uji	Papa líquida	*Leuconostoc mesenteriodes, Lactobacillus platarum*	Quénia e Uganda

7. ESTADO DA ARTE

Comparativamente a outros cereais, como o trigo, o arroz e o milho, o sorgo tem merecido menos atenção em termos de investigação científica. Este facto deve-se à má imagem associada ao sorgo em termos nutricionais, conferindo-lhe o atributo de cereal inferior face aos restantes cereais. Ainda assim, muitos esforços têm sido desenvolvidos no sentido de fomentar a utilização do sorgo na alimentação humana. Vários estudos incorrendo na apreciação da qualidade nutricional do sorgo, ou no incremento da mesma, foram e continuam a ser desenvolvidos, e podem ser divididos em quatro categorias com base nos principais objectivos:

- Análise da composição química do sorgo e caracterização estrutural das suas principais macromoléculas - Estes estudos incidem na composição química do sorgo em termos de hidratos de carbono (amido, açúcares solúveis, fibras), proteínas, polifenóis, lípidos, aminoácidos, vitaminas e minerais. É promovida ainda a caracterização estrutural do amido e das proteínas e o desenvolvimento de técnicas de extracção. (Aarathi *et al.*, 2003, Agudelo *et al.*, 1998, Axtell *et al.*, 1981, Barikmo *et al.*, 2004, Beta *et al.*, 2000, Chibber *et al.*, 1978, Daniel *et al.*, 1966, Dicko *et al.*, 2006, Eggum *et al.*, 1983, El Nour *et al.*, 1998, Elkin *et al.*, 1996, Emmambux e Taylor, 2003, Hamaker *et al.*, 1986, Hamaker *et al.*, 1995, Harbers, 1975, Hibberd *et al.*, 1982, Hoseney *et al.*, 1987, Kurien *et al.*, 1960, MacLean *et al.*, 1981, Miller e Burns, 1970, Mitaru *et al.*, 1985, Nandini e Salimath, 2001, Neucere e Sumrell, 1979, Niba e Hoffman, 2003, Nunes, 2000, Nunes, 2004, Nunes *et al.*, 2004, 2005, Oria *et al.*, 2000, Oria *et al.*, 1995, Price e Butler, 1978, Price *et al.*, 1980, Rom *et al.*, 1992, Shull *et al.*, 1992, Silva e Ciocca, 2005, Sira e Amaiz, 2004, Tanner *et al.*, 1947, Taylor *et al.*, 1984a, Taylor e Schussler, 1986, Taylor *et al.*, 1984b, Taylor *et al.*, 1985b, Verbruggen *et al.*, 1993, Wall e Blessin, 1970, Weaver *et al.*, 1998) . O principal objectivo da maioria destes trabalhos de caracterização estrutural foi contribuir para o entendimento dos factores responsáveis pela diminuição da digestibilidade das proteínas do sorgo com o cozimento.

- Caracterização da flora microbiana presente em alimentos tradicionais fermentados - A flora predominante nos alimentos tradicionais fermentados é bastante complexa, envolvendo a combinação de diversas bactérias, fungos e leveduras. Uma vez que a fermentação é realizada de forma não controlada, nem sempre se conhecem os microrganismos actuantes verificando-se, por vezes, a ocorrência de microrganismos patogénicos. Alguns dos metabolitos produzidos durante a fermentação do sorgo incluem micotoxinas, etil-carbamato e aminas biogénicas, causadores de infecções e intoxicações. Na literatura encontram-se, assim, alguns trabalhos em que é efectuado o isolamento e caracterização da flora microbiana presente em alimentos tradicionais a base de sorgo fermentado, como por exemplo, o *kisra*, o *togwa*, o *mahewu*, o *bushera*, *kunum zaki* e o *hussuwa* (Bvochora *et al.*, 1999, El Nour *et al.*, 1999, Gaffa e Gaffa, 2004, Hamad *et al.*, 1997, Jespersen, 2003, Kunene *et al.*, 2000, Kunene *et al.*, 1999, Mugula *et al.*, 2003b, Mugula *et al.*, 2003c, Muyanja *et al.*, 2003, Pattison *et al.*, 1998).

- Avaliação da qualidade nutricional de alimentos tradicionais - Diversos estudos foram realizados no sentido de avaliar as principais alterações promovidas nos produtos tradicionais cuja preparação envolve a fermentação e/ou a germinação do sorgo. Para tal, efectuaram-se análises aos alimentos tradicionais obtidos no comércio tradicional, ou preparados em laboratório simulando os processos tradicionais de preparação dos mesmos. Os principais parâmetros estudados foram o pH, a acidez titulável, os teores de proteína solúvel e insolúvel, de açúcares totais e redutores, de amido, de aminoácidos, de lípidos e de taninos, a digestibilidade proteica e a digestibilidade do amido (Agu e Palmer, 1999, Ahmed *et al.*, 1996, Akoma *et al.*, 2006, Anibaba e Osagie, 1997, Au e Fields, 1981, Balogun *et al.*, 2006, Balogun *et al.*, 2005, Bond *et al.*, 2005, Bvochora *et al.*, 1999, Chavan *et al.*, 1988, El Tinay *et al.*, 1979, Elkhalifa *et al.*, 2006, Elkhalifa *et al.*, 2004a, Elkhalifa *et al.*, 2004b, Elkhalifa *et al.*, 2005, Elmaki *et al.*, 1999, Erbas *et al.*, 2005, Gadaga *et al.*, 1999, Graham *et al.*, 1986, Hassan e El Tinay, 1995, Ibanoglu *et al.*, 1995, Kazanas e Fields, 1981, Laetitia *et al.*, 2005,

Mahgoub *et al.*, 1999, Moneim *et al.*, 1995, Mugula *et al.*, 2003b, Muyanja *et al.*, 2003, Ogbonna *et al.*, 2003, Ogbonna *et al.*, 2004, Osman, 2004, Sanni *et al.*, 2002, Shayo *et al.*, 2001, Subramanian *et al.*, 1995, Tou *et al.*, 2007, Traoré *et al.*, 2004, Uvere *et al.*, 2000, Yousif e El Tinay, 2001).

- Desenvolvimento de produtos à base de sorgo - Com o objectivo de satisfazer a procura dos consumidores, muitos alimentos à base de sorgo têm sido desenvolvidos em laboratório de modo a se obter produtos mais seguros em termos microbiológicos e com maior valor nutricional. Alguns destes alimentos são desenvolvidos de acordo com o processo tradicional de fermentação (por exemplo, *ogi*, *boza*, *togwa* e *pito*), mas com adição de "starters" seleccionados (microrganismos propagadores), isolados da flora endógena destes produtos (Mugula *et al.*, 2003a, Mugula *et al.*, 2003c, Orji *et al.*, 2003, Teniola *et al.*, 2005, Zorba *et al.*, 2003). Noutros casos, promove-se o desenvolvimento de alimentos de sorgo, na sua maioria alimentos complementares para lactentes, testando-se diferentes formulações (Ibanoglu *et al.*, 1995, Mahgoub, 1999, Nnam, 2001, Thaoge *et al.*, 2003) ou, no caso de alimentos fermentados, testando-se diferentes culturas de microrganismos (Lalude e Fashakin, 2006, Nout e Ngoddy, 1997, Onilude *et al.*, 1999, Sanni *et al.*, 1999a, b). Em ambos os casos o produto final é analisado segundo alguns dos parâmetros descritos no ponto anterior, e comparados com os produtos desenvolvidos tradicionalmente ou com produtos comerciais (como por exemplo papas "Cerelac" ou papas da "Nestlé").

CAPÍTULO 2 – OBJECTIVOS

1. ENQUADRAMENTO DO TRABALHO

Devido à escassez de alimentos de origem animal, a maioria das populações Africanas depende dos cereais, entre os quais o sorgo, para a sua alimentação. Os alimentos de sorgo, sobretudo as papas, são consumidos em várias partes do mundo, particularmente nos países subdesenvolvidos, onde representam a base da dieta. O *ogi* e o *ugi* são exemplos de papas de sorgo utilizadas na alimentação de adultos e crianças e que se encontram enraizadas nos hábitos alimentares do povo Africano.

Na preparação destes produtos são empregues processos tradicionais que normalmente envolvem a fermentação e /ou a maltagem do sorgo. Estes processos de preparação passam de geração em geração e apresentam uma importância cultural em diversos países da África e da Ásia, sendo práticas familiares que se mantêm ao longo de séculos. Utilizam matérias-primas produzidas localmente, apresentam baixos custos e constituem parte integrante da rotina dos habitantes de diversas aldeias.

Para além das questões culturais, a fermentação e a maltagem assumem especial importância na medida que contribuem para a melhoria da qualidade nutricional do sorgo, aspectos que serão abordados em pormenor no próximo capítulo. Dado que o consumo de sorgo está associado aos elevados índices de subnutrição das populações onde o seu consumo é elevado, o estudo aprofundado de formas de processamento que possam contribuir para um incremento nutricional torna-se um assunto de suma importância no âmbito da segurança alimentar em África.

A fermentação e a maltagem são, no entanto, formas de processamento bastante dispendiosas em termos de tempo, o que se traduz num inconveniente, sobretudo nos meios urbanos.

Como consequência da urbanização, os hábitos alimentares sofrem alterações resultantes da falta de tempo para a confecção de alimentos tradicionais. Em estudos realizados no maior centro urbano da província de Limpopo (África do Sul), quando interrogados sobre as necessidades de novos produtos, verificou-se que existe entre os seus habitantes uma procura por biscoitos e papas instantâneas a base de sorgo, do tipo "Purity" ou "Nestum", bem como por iogurtes com adição de sorgo. Os consumidores clamam por produtos de rápida preparação que possam ser utilizados, por exemplo, ao pequeno-almoço (Bichard *et al.*, 2005).

Os mesmos estudos revelaram que, apesar de viverem numa zona urbana, os habitantes de Limpopo mantêm os hábitos de consumo de sorgo. O almoço normalmente é realizado no local de trabalho, e normalmente inclui pães de sorgo feitos em casa. Alguns habitantes almoçam em restaurantes, sendo o prato principal à base de carne ou vegetais acompanhados das papas de sorgo. As papas líquidas são consumidas ao desjejum diariamente, várias vezes por semana ou apenas aos fins-de-semana, consoante o tempo disponível para a sua preparação. Podem ser ácidas ou de sabor regular. As papas ácidas requerem a fermentação do sorgo. A farinha de sorgo é adquirida no comércio tradicional e fermentada em água durante a noite, o que requer bastante tempo de preparação. Por esta razão, algumas pessoas adicionam produtos acidificantes em substituição da fermentação, como por exemplo, maionese, vinagre, extracto de tamarindo ou sumo de limão. Dependendo da disponibilidade financeira das famílias, as papas líquidas podem ainda ser complementadas com leite, manteiga de amendoim ou margarina. As papas sólidas são menos consumidas que as líquidas. Normalmente são ingeridas ao almoço e ao jantar, acompanhadas de carne ou leite fermentado, e em cerimónias fúnebres e casamentos. Outra forma bastante popular de consumo de sorgo é a cerveja feita em casa (Bichard *et al.*, 2005).

Com a industrialização e crescente urbanização, torna-se assim necessária a produção em larga escala de alimentos de sorgo culturalmente aceites, de preparação instantânea e acessíveis à maioria da população, de modo a satisfazer a procura dos consumidores.

Por outro lado, os alimentos fermentados de sorgo constituem um grupo de alimentos que é produzido a nível caseiro, em aldeias e em pequenas indústrias, sendo a microbiologia dessas fermentações bastante complexa. A microflora do sorgo varia de aldeia para aldeia e, dentro da mesma aldeia, de família para família, envolvendo por vezes a combinação de diversas bactérias, fungos e leveduras que nem sempre são conhecidas. Como consequência, verificam-se grandes variações no produto final e, por vezes, problemas de contaminações associados à proliferação de microrganismos patogénicos. Os principais factores de risco incluem a utilização de matérias-primas contaminadas, a ausência de pasteurização e condições de fermentação pouco controladas, o que pode dar origem a infecções e intoxicações provocadas por metabolitos microbianos produzidas durante a fermentação. A obtenção de um produto microbiologicamente seguro e de qualidade consistente, constitui assim mais um motivo impulsionador do desenvolvimento de técnicas de processamento do sorgo adaptáveis à escala industrial.

A produção industrial de alimentos de sorgo, para além das referidas vantagens em termos de diminuição dos tempos de preparação, de melhoria da qualidade nutricional e de segurança alimentar, contribui ainda para o aumento da procura por sorgo cultivado localmente, diminuindo assim as necessidades de importação de outros produtos, como por exemplo do trigo, em África (Hamaker, 2007). O processamento comercial do sorgo em alimentos e bebidas de valor acrescentado pode, assim, vir a ser o veículo condutor do desenvolvimento económico em África (Taylor *et al.*, 2006).

O *ogi* é um exemplo já existente de produto tradicional fermentado que foi convertido a uma escala semi-industrial. O processo industrial de fermentação é desenvolvido com a flora endógena, que se supõe ser constituída por *Lactobacillus plantarum*, *Candida krusei* e *Debaromyes hansenii*. O "*soy-ogi*" é um alimento desenvolvido pelo FIIRO ("Federal Institute of Industrial Research"), em que a soja foi adicionada ao *ogi*, com o intuito de melhorar a sua qualidade nutricional e o conteúdo proteico.

Outro exemplo é o da Cerveja Sul-Africana. A Cerveja Sul-Africana é uma cerveja de sorgo (tal como o *pito* e o *burukutu*), acastanhada, de sabor ácido, aparência opaca e consistência de

papa líquida (Achi, 2005). A produção de cervejas de sorgo envolve dois tipos de fermentação: fermentação láctica e fermentação alcoólica. A fermentação láctica confere-lhe o sabor ácido, característica necessária ao armazenamento, e garante a qualidade do produto final. Quando o pH da cerveja é de aproximadamente 3,5, a mistura é fervida para matar todos os microrganismos na forma vegetativa e interromper a fermentação láctica. A fermentação alcoólica inicia-se então por adição de leveduras da espécie *Saccharomyces cerevisiae* (Pattison *et al.*, 1998). A sua comercialização é feita em embalagens de cartão ou plástico com um orifício que permite a libertação do dióxido de carbono produzido na fermentação (Pattison *et al.*, 1998).

2. OBJECTIVOS PROPOSTOS

Tendo em conta a importância do sorgo na dieta alimentar das populações da África e da Ásia e, por outro lado, as suas fracas características nutricionais, torna-se crucial estudar os factores associados à baixa digestibilidade, especialmente em produtos cozinhados, considerar a qualidade nutricional do sorgo e propor novas formas de a incrementar.

Este trabalho tem assim como principal objectivo sugerir uma forma de processamento de sorgo adaptável à escala industrial que, sendo culturalmente aceite e sensível aos hábitos alimentares, aos factores sociais e às limitações económicas e tecnológicas das populações a que se destina, possa dar origem a um produto alimentar seguro e enriquecido em termos nutricionais.

Para tal, pretendia-se:

- Estudar as alterações promovidas nas proteínas, no amido e nos lípidos resultantes de diferentes formas de processamento;
- Promover um estudo do processo fermentativo de sorgo avaliando os efeitos promovidos por diferentes espécies microbianas;

- Propor uma preparação alimentar à base de sorgo com características nutricionais melhoradas no que respeita ao balanço em aminoácidos essenciais, à digestibilidade da proteína e do amido e à viscosidade do produto final.
- Estabelecer uma plataforma de conhecimentos que permita interactuar e participar em projectos internacionais em parceria com países terceiros.

Este trabalho pretende dar continuidade ao projecto "Improvement of the Protein Quality of Sorghum and its Introduction into Staple Food Products for Southern and Eastern Africa"(Programa INCO IC18-CT96005), aos trabalhos de mestrado intitulados "Contribuição para a caracterização da fracção de prolaminas de duas variedades de sorgo" (Nunes, 2000) e "Efeito do Processo Fermentativo em Farinha de Sorgo" (Correia, 2004), e ao trabalho de doutoramento intitulado "Estudo das Interacções entre componentes da farinha de Sorghum bicolor (L.) Moench" (Nunes, 2004).

CAPÍTULO 3 – ESTUDO DAS ALTERAÇÕES PROMOVIDAS POR DIFERENTES FORMAS DE PROCESSAMENTO

1. INTRODUÇÃO

As formas de processamento aplicadas à preparação de produtos de sorgo normalmente envolvem calor seco ou húmido (Murty e Kumar, 1995), germinação e fermentação (Gadaga *et al.*, 1999).

O cozimento da farinha de sorgo em água é utilizado na preparação das papas e de alimentos substitutos do arroz. No entanto, tal como foi referido no capítulo 1, o cozimento promove alterações estruturais nas proteínas do sorgo que resultam na diminuição da digestibilidade proteica. Em termos nutricionais, esta é a principal característica que distingue o sorgo dos outros cereais e da qual resulta a má imagem que lhe está associada.

De forma a reverter os efeitos deletérios do cozimento, torna-se necessário recorrer a outras formas de processamento que incrementem a qualidade proteica deste cereal. Esta é provavelmente a razão pela qual, em África, muitos dos alimentos a base de sorgo são fermentados antes de serem consumidos (Board on Science and Technology for International Development, 1996).

As principais alterações que se observam com a fermentação compreendem um aumento no teor de aminoácidos, a ruptura das proteínas e a destruição de quaisquer inibidores das enzimas digestivas que possam estar presentes.

A fermentação promove uma melhoria na qualidade proteica pela conjugação de dois factores. Por um lado, verifica-se uma melhoria da composição em aminoácidos essenciais, resultante da biossíntese por parte dos microrganismos (Au e Fields, 1981, Chavan e Kadam, 1989b, Chavan *et al.*, 1988, Kazanas e Fields, 1981, Sanni *et al.*, 2001). Por outro lado, verifica-se ainda um aumento da digestibilidade proteica pela acção de enzimas microbianas (Chavan *et al.*, 1988, Hassan e El Tinay, 1995, Moneim *et al.*, 1995). As enzimas microbianas clivam as proteínas de reserva resultando na sua conversão em proteínas solúveis e aminoácidos, mais acessíveis ao ataque enzimático (Chavan *et al.*, 1988, Taylor e Taylor, 2002, Yousif e El Tinay, 2001).

O aumento da digestibilidade proteica com a fermentação foi verificado em vários alimentos tradicionais de sorgo, como por exemplo no *kirsa* e *abrey* (Axtell *et al.*, 1981), no *nasha* (Graham *et al.*, 1986) e, mais recentemente, no *khamir* (Osman, 2004).

Para além das proteínas solúveis, a fermentação espontânea do sorgo promove um incremento no teor de proteínas insolúveis como consequência da degradação dos hidratos de carbono (Chavan e Kadam, 1989a, b, Chavan *et al.*, 1988, Taylor e Taylor, 2002) e do crescimento microbiano (Shayo *et al.*, 2001).

A fermentação contribui para o incremento nutricional não apenas pela melhoria da qualidade proteica, mas também por alterações promovidas noutros componentes da farinha. Entre as quais, verifica-se um aumento nos açúcares totais e redutores, melhoria da digestibilidade do

amido e diminuição do amido resistente (El Tinay *et al.*, 1979, Elkhalifa *et al.*, 2006, Elkhalifa *et al.*, 2004a, Elkhalifa *et al.*, 2004b, Hassan e El Tinay, 1995, Kazanas e Fields, 1981).

Estas alterações devem-se à acção de amilases microbianas que clivam o amido em açúcares livres que poderão ser utilizados pelos microrganismos como fonte de energia. Alguns estudos sugerem ainda que a melhoria da digestibilidade do amido está associada ao ataque proteico (Elkhalifa *et al.*, 2006, Elkhalifa *et al.*, 2004a). Segundo estes autores, na farinha não fermentada as proteínas do endosperma restringem a acessibilidade ao amido, impedindo a sua completa gelatinização. A hidrólise das proteínas localizadas nas camadas mais externas da matriz proteica, na qual os grânulos de amido se encontram aprisionados, permite a libertação dos mesmos. Ao clivarem as proteínas, os microrganismos do meio fermentativo facilitam o acesso das enzimas digestivas ao amido.

Os produtos fermentados são mais ricos em vitaminas, sintetizadas pelos microrganismos fermentativos, e os seus minerais apresentam uma maior biodisponibilidade. Gazzaz *et al.* (1989) mostraram ocorrer síntese de vitamina B-12 durante o processo de fermentação do sorgo. El Tinay *et al.* (1979) observaram ainda um grande aumento dos teores de tiamina e niacina no sorgo fermentado para a produção do *kisra*. Resultados semelhantes foram obtidos por Au e Fields (1981) e Kazanas e Fields (1981).

Estudos com duas espécies de sorgo, revelaram ainda uma diminuição no teor de taninos (Hassan e El Tinay, 1995, Osman, 2004). De acordo com Bach Knudsen e Munck (1985), a fermentação também inibe a ligação dos taninos às proteínas. O ácido fítico é outro factor anti-nutricional que diminui com a fermentação (Osman, 2004).

A fermentação permite ainda um incremento das características organolépticas, da textura, do tempo de vida dos alimentos e uma diminuição dos tempos de cozimento (Jespersen, 2003).

Em muitos alimentos tradicionais, a adição de malte de sorgo é utilizada para iniciar a fermentação espontânea (Lorri e Svanberg, 1995, Steinkraus, 1995). A maltagem, processo comum nas áreas onde o sorgo é produzido, pressupõe deixar que o grão germine e brote. Um exemplo de produto cuja preparação envolve a fermentação e a germinação é o *hulu-mur*.

O sorgo é germinado para a preparação de cervejas, papas e outros pratos tradicionais, sendo outra forma de processamento que permite incrementar as características nutricionais dos alimentos. A germinação promove alterações notáveis na qualidade nutricional de um cereal. Wang e Fields (1978) verificaram que a germinação do sorgo aumenta o valor nutricional (VN) de 54,6 % para 63,0 %, e a relação de eficiência de proteínas (REP), de 1,5 para 1,7 %.

O principal objectivo da germinação é o desenvolvimento de enzimas hidrolíticas que não estão presentes no grão não germinado. Com a germinação, verifica-se a activação de enzimas intrínsecas, como por exemplo de proteases, amilases, fitases e enzimas que degradam fibras (Taylor *et al.*, 1985b).

As proteases intrínsecas, presentes no grão germinado, hidrolizam as proteínas de reserva do endosperma em polipeptídeos, peptídeos e aminoácidos. Segundo Subramanian *et al*. (1995) as prolaminas e glutelinas são clivadas em proteínas mais simples, solúveis. Resultados semelhantes foram obtidos por Ogbonna *et al*., (2003) que verificaram um aumento no teor de aminoácidos e proteínas solúveis em água.

Como consequência do ataque aos corpos proteicos e à matriz proteica, as proteínas tornam-se mais acessíveis ao ataque enzimático, verificando-se um aumento da digestibilidade proteica (Elkhalil *et al.*, 2001, Elmaki *et al.*, 1999, Moneim *et al.*, 1995, Taylor *et al.*, 1985a). Com a germinação verifica-se também um incremento no balanço de aminoácidos, com um aumento no teor de lisina, metionina e triptofano (Wang e Fields, 1978, Wu e Wall, 1980).

Entre as amilases produzidas no processo de germinação do grão encontra-se a α-amilase, uma enzima conhecida pela sua capacidade de liquefazer alimentos amiláceos transformando amido insolúvel em açúcares solúveis (dextrinas e maltose). Esta característica é especialmente útil na preparação de alimentos complementares ao leite materno. Como foi referido no capítulo 1, a baixa densidade energética e nutriente dos alimentos complementares a base de sorgo é o agente etiológico da malnutrição em crianças (Michaelsen, 1998). A utilização de malte de sorgo na preparação das papas permite diminuir a viscosidade de papas altamente concentradas, aumentando a densidade energética e nutriente. Daqui surge o conceito de "Amilase-Rich Flour" (ARF) ou "Flour Power" (FP) (Belton e Taylor, 2004). A

energia que as crianças pequenas podem consumir está muitas vezes limitada ao volume de alimento que podem ingerir. O tratamento enzimático realizado pela adição de pequenas quantidades de malte de sorgo (ARF) às papas acabadas de fazer, resulta na obtenção de alimentos mais concentrados em energia e proteínas para crianças de certas idades (Traoré *et al.*, 2004).

Os teores de ácido fítico sofrem uma diminuição pela acção das fitases desenvolvidas no processo germinativo (Elkhalil *et al.*, 2001). Elmaki *et al.*, (1999) verificaram ainda uma diminuição nos taninos, nas fibras e nos lípidos.

Com a germinação consegue-se ainda incrementar o teor em vitaminas do sorgo. As vitaminas C e B12 são sintetizadas e verifica-se um aumento do conteúdo de riboflavina (Mahgoub *et al.*, 1999, Taur *et al.*, 1984).

Outra forma de processamento do sorgo é a pipocagem. As pipocas de sorgo são consumidas como snacks na Índia e na África. Na pipocagem, os grãos de sorgo são aquecidos num recipiente fechado sob calor intenso. Esta forma de processamento consiste num tratamento térmico, tal como o cozimento, mas na ausência de água. Existem poucos estudos sobre a pipocagem do sorgo. No entanto, sabe-se que apesar de também envolver aquecimento, a pipocagem não produz efeitos adversos na digestibilidade proteica, contrariamente ao cozimento.

2. OBJECTIVOS

De modo a propor novas formas de processamento do sorgo para consumo humano, é importante compreender melhor os mecanismos pelos quais os métodos tradicionais de preparação dos alimentos à base de sorgo exercem influência sobre as proteínas, o amido e os lípidos da variedade a estudar. O conhecimento destes mecanismos poderia contribuir para uma melhor compreensão dos fenómenos resultantes das fermentações controladas a serem realizadas posteriormente.

Este capítulo teve assim como principal objectivo efectuar um estudo comparativo dos efeitos promovidos por diferentes formas de preparação.

3. ENQUADRAMENTO EXPERIMENTAL

Numa primeira parte deste capítulo estudaram-se os efeitos promovidos pelas principais formas de preparação de alimentos tradicionais de sorgo: cozimento, fermentação, germinação e pipocagem.

Outras formas alternativas de processamento do sorgo, que poderiam resultar em efeitos interessantes para a qualidade deste cereal, continuam por estudar. Um exemplo é o aquecimento em banho-maria que, tal como a pipocagem, é um processo de aquecimento por calor seco. Com o objectivo de compreender melhor a razão pela qual o cozimento e a pipocagem apresentam efeitos distintos na digestibilidade proteica; e de perceber até que ponto o papel da água poderá ser relevante; optou-se assim por incluir o banho-maria neste estudo comparativo.

A alta pressão é uma tecnologia alternativa ao processamento térmico que promove alterações nas propriedades funcionais dos alimentos sem promover alterações na sua composição e no sabor. O processamento por alta pressão é uma técnica promissora que nunca foi aplicada ao sorgo e cujos efeitos se desconhecem.

Por esta razão, posteriormente considerou-se importante investigar se a aplicação da alta-pressão ao sorgo poderia reverter os efeitos deletérios promovidos pelo cozimento na digestibilidade proteica. Realizaram-se assim ensaios preliminares, em que se verificou que a aplicação de altas-pressões promovia um aumento na digestibilidade proteica, tanto do sorgo cru como do sorgo cozido. Com o objectivo de optimizar as condições de aplicação da alta-pressão ao sorgo, foram então desenvolvidos novos ensaios em que se avaliaram os resultados obtidos com diferentes níveis de pressão e variando os tempos de processamento das amostras.

Um dos objectivos gerais deste trabalho foi avaliar as vantagens da utilização de malte de sorgo nas fermentações. Deste modo, neste capítulo realizou-se ainda o acompanhamento do

processo germinativo ao longo do tempo com o objectivo de conhecer o tempo óptimo de germinação para os fins pretendidos.

4. COMPARAÇÃO DOS EFEITOS PROMOVIDOS NO SORGO POR DIFERENTES FORMAS DE PROCESSAMENTO

"Comparison of the Effects Induced by Different Processing Methods on Sorghum Proteins"

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Comparison of the Effects Induced by Different Processing Methods on Sorghum Proteins

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Abbreviations Used

FT-IR – Fourier Transform Infrared

HMW –High Molecular Weight

LV - latent variables

PCA – Principal Components Analysis

PLS – Partial Least Squares regression

RMSEC - Root Mean Square Error of Calibration

SDS-PAGE – Sodium Dodecyl Sulfate Polycrylamide Gel Electrophoresis

SNV - Standard Normal Variate

t-butanol – 2-methyl-propan-2-ol

ABSTRACT

The effects induced by cooking, dry heating, popping, fermentation and germination on sorghum proteins were studied. Changes promoted by these processing methods were compared via sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE), *in vitro* protein digestibility (IVPD) assay and Fourier Transform Infrared (FT-IR).

Cooking decreased protein digestibility and extractability. With dry heating, a decrease of only 4% in protein digestibility was observed. In these samples protein extractability was not affected. Popping had no effect on protein digestibility, however, it was noticed a decrease of protein extractability of popped samples. Fermentation and germination promoted an increase of 39.6% and 20.8% in the protein digestibility, respectively, and an increase in protein extractability.

FT-IR analysis and multivariate correlation (PLS1) showed a calibration of protein digestibility values allowing quantification of unknown samples.

As dry heated samples have practically the same digestibility values as unprocessed samples, we concluded that water plays an important role on deleterious effects of cooking.

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the major food crops in the semiarid regions of Africa and Asia (Aboubacar et al., 1990). This cereal is the main staple food for the world's poorest people and is consumed in different traditional forms in various geographic areas (Eggum et al., 1982).

Sorghum is processed into a wide array of foods. Processing methods applied to the preparation of sorghum products usually involves wet or dry heat (Murty and Kumar, 1995), germination and fermentation (Gadaga et al., 1999). These processing methods promote modifications in sorghum protein structure affecting its nutritional quality.

It has been shown that, when sorghum is submitted to cooking, some changes occur in the protein fraction, reducing its digestibility (Nunes et al., 2004). Some authors suggest that cooking promotes the formation of disulphide-bonded protein polymers, leading to a change in protein structure (Hamaker et al., 1986). This is believed to induce the reduction of protein digestibility in cooked sorghum products.

As opposed to wet cooking, processing methods such as germination and fermentation alters protein structure to make it more digestible and bioavailable.

Germination causes activation of intrinsic amylases, proteases, phytases and fiber-degrading enzymes, thereby increasing nutrient digestibility (Taylor et al., 1985). According to Elkhalil et al. (2001), the activity of intrinsic proteases in germinated seeds leads to an increase in *in vitro* protein digestibility. Several indigenous fermented foods prepared from sorghum are common in India and many parts of Africa. Taylor and Taylor (2002) found that sorghum fermentation increases insoluble protein digestibility, suggesting that fermentation causes

structural changes in sorghum storage proteins (prolamins and glutelins), making them more accessible to pepsin attack. According to Yousif and El Tinay (2001), this increase in protein digestibility can be attributed to the partial degradation of complex storage proteins into more simple and soluble products.

Popped sorghum, consumed as a snack, is traditionally prepared by dry heating grain in a hot pan or bowl over a steady fire (Murty and Kumar, 1995). Parker et al. (1999) reported that dry thermal treatment in the form of popping does not decrease sorghum protein digestibility, unlike cooked sorghum. These authors suggested that popping is an explosive process that leads to a fragmentation of the cell walls of the vitreous endosperm. This effect appeared to improve the accessibility of the starch and protein components of the endosperm foam to enzymes in the digestive tract which is beneficial for protein digestibility. Duodu et al. (2001) found that the extent of secondary structural changes promoted by popping is lower than the promoted by wet cooking.

Until now, no studies have compared the changes that take place in sorghum proteins when the same sorghum variety is submitted to different processing methods. Due to the distinct and not well characterized effects induced by the common used processing methods, this study sought to compare the changes in protein structure and digestibility induced by cooking, dry heating, germination, fermentation, and popping using electrophoresis, FT-IR and pepsin *in vitro* protein digestibility assay

2. Materials and methods

2.1. Sorghum grain

Sorghum bicolor (L.) Moench of Jumbo variety from Australia was purchased in the retail market and stored at 4ºC. The characterization of the reddish brown grains showed that this sorghum is non-tannin (Type I) variety (determined by the chlorox bleach test described by Waniska et al., 1992), has 9% of total protein (determined by the Kjeldahl method described by Nunes, 1998) and 0.32% of polyphenols content (determined by the method described by Price and Butler, 1977).

2.2. Processing methods

For cooking, dry heating and fermentation procedures, whole grain samples were ground with a coffee mill to pass through a 0.4 mm sieve.

Cooked samples were obtained by mixing 1 part of ground flour with 10 parts of water. The mixture was placed in a boiling water bath for 20 minutes. After cooking, the samples were freeze-dried and ground again. For dry heated sorghum, flour was placed in a capped glass tube and kept in a boiling water bath for 90 minutes to ensure that temperature transfer was efficient.

Sorghum flour was fermented according to the traditional method. Fermentation was carried out by mixing sorghum flour (5 g) with distilled water (1:2 wt/vol) to obtain a thick paste. Then, previously fermented dough (2 g) was used as a starter. The mixture was left to ferment with the natural microflora for 2 days at room temperature (*ca.* 25ºC) to achieve pH 4. After fermentation, the sample was freeze-dried and ground again.

For germination, grains were manually cleaned with water followed by ethanol 70% (v/v) and, then, hypochlorite solution of 1% (v/v) available chlorine. Subsequently, the grains were rinsed several times with sterilized water. This treatment was applied to prevent mold growth. The washed grains were then soaked in sterilized water for 18 hours. At the end of soaking, the grains were drained and spread over filter paper on pre-sterilized trays. Germination was carried out in an oven at 26 ºC for 7 days and turning the malt twice a day to avoid excessive root malting. To avoid dryness the sample was sprayed, twice a day, with sterilized water. On day seven, 100 g sample of grain were taken for subsequent analysis. The sample was dried in a vacuum oven and derooted. Finally, the grains were ground in a coffee mill and sieved to obtain a 0.4 mm granulometry.

Whole sorghum grains were popped in a domestic hot-air popcorn maker. Popped grains were then ground and sieved as described previously.

2.3. In vitro protein digestibility determination

Unprocessed and processed samples were subjected to *in vitro* protein digestion using pepsin (Sigma - P-7000 - 975 U/mg protein) as described by Nunes et al. (2004)**.** Flour samples (60 mg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 M KH_2PO_4 pH 2 buffer) in a water bath (37^{o}C) for 0 (control sample) and 120 minutes. After this period of time, the digestions were stopped by the addition of 100 μL of 2 M NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, 25ºC) for 3 minutes and the supernatants discarded. The residues were washed with 1 mL of 0.1 M K_2HPO_4 pH 7 buffer, centrifuged and washed again with 1 mL of water. The residues with undigested proteins were then freeze-dried. The N content that remained in the control sample and after

120 minutes of digestion was determined using the Kjeldahl method described by Nunes (1998). The percentage of digested protein, calculated by difference between content of total N in control sample and after 120 minutes of digestion, was defined as *in vitro* protein digestibility.

2.4. Proteins analysis by SDS-PAGE

In order to extract kafirins, 100 mg of each uncooked and processed flours were extracted with 0.5 mL of *t*-butanol 60%, with mechanical stirring. As the aim of this study was to follow proteins in their non-reduced stage (both polymers and monomers), reducing agents were not used in this extraction. The extraction time of the uncooked, dry heated, fermented and germinated samples was 1 h. Effective extraction of kafirins from cooked and popped sorghum samples was only possible after a 24 h extraction. In case of popped sample, an additional extraction procedure, with 0.5mL 0.0125M $Na_2B_4O_7.10H_2O$ (pH10), 2% (m/v) SDS and 1% (v/v) 2-mercaptoethanol, was applied (Wallace et al., 1990). After extraction, the mixtures were centrifuged (2500 x g, 25 ºC) for 3 minutes.

For SDS-PAGE, 20 μL of *t*-butanol protein extract was dried under N2 atmosphere and the obtained residue was dissolved in electrophoretic sample buffer [2% (w/v) SDS, 0.0625 M Tris, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, pH 6.8]. To observe the oligomers, nonreducing conditions were applied. When reducing conditions were needed, 5% (w/v) 2-ME was used in the sample buffer. The samples were heated, for 5 minutes, in a boiling water bath and 7 μL were applied in a 15% acrylamide SDS-PAGE gel (Laemmli method). The gel was run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad - Hercules, USA). Electrophoresis was conducted at 150 V for 1.5 h until the tracking dye, bromophenol blue,

reached the bottom of the resolving gel. Molecular weight markers were used to compare electrophoretic mobility of proteins (Broad Range Markers – BioRad). Gels were stained with Coomassie Blue R (GE Healthcare - Uppsala, Sweden) and destained with 40% methanol and 10% acetic acid (Shewry et al., 1995).

2.5. Analysis of SDS-PAGE images

Electrophoretic gels were digitalized without previous drying, in a Hewlett Packard ScanJet 3600C scanner (California USA). The acquired image was converted into a matrix representative of the different colors intensities (color-coded images) (Nunes, 2004).

Each one of the digitalized electrophoretic lanes was selected, cut from the electrophoregram and submitted to a mathematical treatment based on joint density probability estimation (Barros et al., 1997). As result, difference and independence matrices were obtained. The independence matrix corresponds to a noiseless color-coded image. The profile recovered from each image corresponds to its maximum value.

This mathematical treatment acts as a filter that enhances protein concentration determination.

2.6. FT-IR

Fourier Transform Infrared (FT-IR) spectroscopy was used to evaluate the effects of processing methods. The FT-IR spectra were obtained covering with the sample the 2x2 mm diamond crystal from a Golden Gate single reflection ATR system in a Bruker IFS-55 spectrometer (Ettlingen, Germany). The spectra were recorded in absorbance mode from 4000 to 500 cm-1, co-adding 128 scans at 8 cm^{-1} resolution. Five replicates were collected for each sample. The spectra obtained were transferred into a data analysis package (Barros, 1999). For Principal Component Analysis (PCA), the 1780-800 cm^{-1} region was selected as it comprises

lipids (1743 cm^{-1}), protein (1650 – 1500 cm^{-1}) and starch signals (around 1000 cm^{-1}), major components of sorghum flour. Each spectrum was SNV corrected (Standard Normal Variate). The PCA allowed the characterization of the sample relationships (scores plans) and the recovery of their sub-spectral profiles (loadings) (Jolliffe, 1986).

For multivariate calibration FT-IR spectra in the range of 1800-1477 cm^{-1} were imported into in-house developed routines for Partial Least Squares regression (PLS1) (Helland, 2001; Martens, 2001; Wold et al., 1983; Wold et al., 2001).

3. Results and discussion

3.1. in vitro protein digestibility results

Table 1 shows the *in vitro* protein digestibility values of all sorghum samples subjected to the different processing methods. In previous studies, the cooking procedure promoted a decrease of sorghum protein digestibility (Axtell et al., 1981; Eggum et al., 1983; Mitaru et al., 1985; Oria et al., 1995). In the sorghum variety studied in this work, cooking promoted a protein digestibility decrease from 53% to 30%, which corresponds to a drop of 43%. When the heating was developed under dry conditions, as it occurred in the dry heated sample, the protein digestibility value suffered a small decrease of 4%. This result suggests that, besides protein crosslinking, the gelatinization that occurred in wet cooking sample, played an important role on the access of digestive enzymes to storage proteins. This conclusion is supported by Duodu et al. (2002) that previously found that cooked sorghum flour samples treated with alpha-amylase had higher protein digestibility than cooked samples without alpha-amylase treatment, suggesting that gelatinized starch affects protein digestibility.

Table 1 – *In vitro* protein digestibility of unprocessed, cooked, dry heated, popped, fermented and germinated samples.

Samples processing	Digestibility Values (%)[1,2]
Unprocessing	53.3 ± 0.3 c
Cooking	30.4 ± 0.2 a
Dry heating	50.7 ± 0.4 b
Popping	53.8 ± 0.4 c
Fermenting	74.4 ± 0.6 e
Germinating	63.9 ± 0.3 d

[1] Mean of three replicates.
[2] Means with different letters in same column are significantly different ($p < 0.05$)

However, the digestibility of popped samples did not decrease. It is important to highlight that popping is another type of dry heat procedure but with a totally different mechanism. Our results are in agreement with previous one (Parker et al., 1999) that claim that popping does not decrease sorghum protein digestibility values. According to these authors, cell wall fragmentation induced by popping improves the accessibility of storage protein to digestive enzymes. Additionally, other studies showed that the increase on β-sheet components provided by thermal processing occurs to a greater extent by wet cooking than by popping (Duodu et al., 2001).

We also confirmed that both germination and fermentation increase protein digestibility values due to the presence of endogenous and microbial enzymes. The digestibility values increased from 53% to 74% and to 64% with fermentation and germination, respectively.

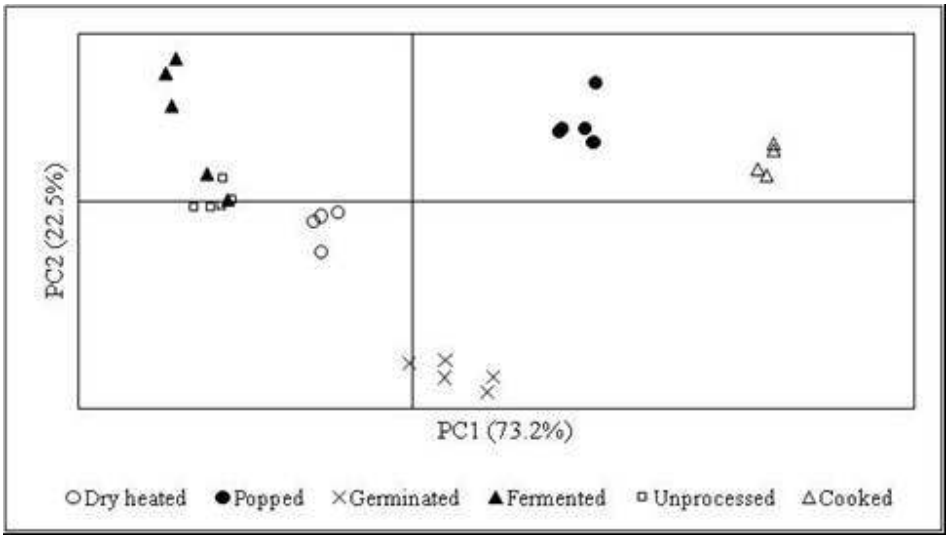
3.2. FT-IR results

By PCA analysis of the FT-IR spectra, we can characterize how processing methods affect proteins, lipids and starch of sorghum samples. The scores scatter plot of all samples (Figure 1a) showed that cooked and popped samples are separated from dry heated, fermented and unprocessed samples, along the PC1 axis. In the loadings profile (Figure 1b), this distinction is due to a starch absorption band at 1022 cm^{-1}, and is a consequence of gelatinization and rupture of the starch granules by the cooking and popping procedures, respectively. The structural changes promoted by starch gelatinization can negatively influence protein digestibility by decreasing enzyme accessibility. In fact, one can also detect the decrease of the 1639 and 1527 cm^{-1} bands, ascribed to proteins, which are located in PC1 negative (Figure 1b), and are negatively correlated to popped an cooked samples (Figure 1a) indicating that the protein fraction of this samples are less visible in FT-IR spectra. The two samples in PC1 (+) has gelatinized starch that overlap protein fraction. The presence of the popped samples in the same quadrant of the cooked samples (Figure 1a) suggests that starch is largely affected in the popping explosive processing. Although starch structure may be strongly affected, protein digestibility was not affected (Table 1). The proximity of unprocessed and dry heated samples in score scatter plot (PC1 (-)) (Figure 1a), suggests that changes promoted by dry heat treatment has no significant influence on lipids and slight influence in proteins (as is shown by the digestibility results in Table 1). Unprocessed, fermented and dry heated samples are

characterized by signals from lipids (1743 cm^{-1}), proteins (1639 and 1527 cm^{-1}) and starch (971 cm^{-1}) (Figure 1a and b). The presence of the germinated sample around the PC1 axis origin (Figure 1a) shows that, this sample, has a weak influence of PC1 loadings profile (Figure 1b).

PCA also separates fermented, popping, cooked and unprocessed samples from germinated samples along the PC2 axis (Figure 1a). In the loadings profile (Figure 1b), germinated samples, are characterized by a signal at 979 cm^{-1}, characteristic of starch structural changes and a decrease of the 1743 cm^{-1}, attributed to lipids (triglycerides). In germinated samples, this structural change can be due to the starch chain shortening by endogenous amylases.

a)



b)

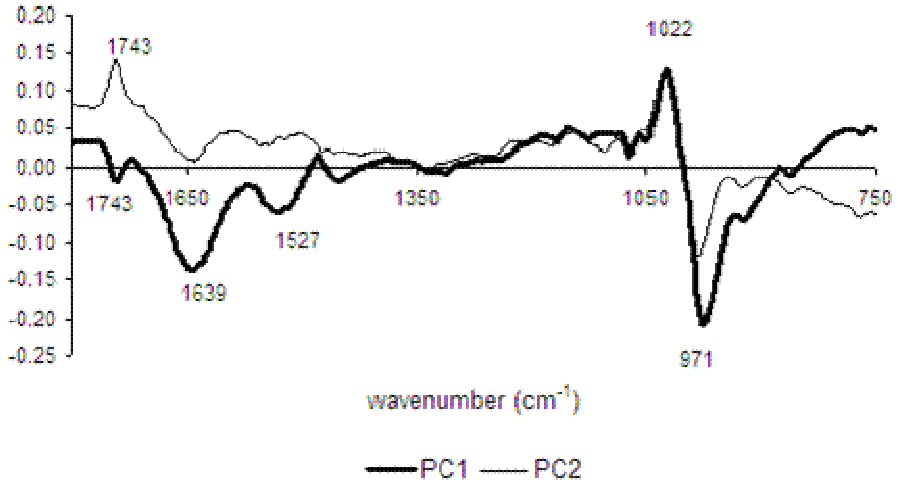
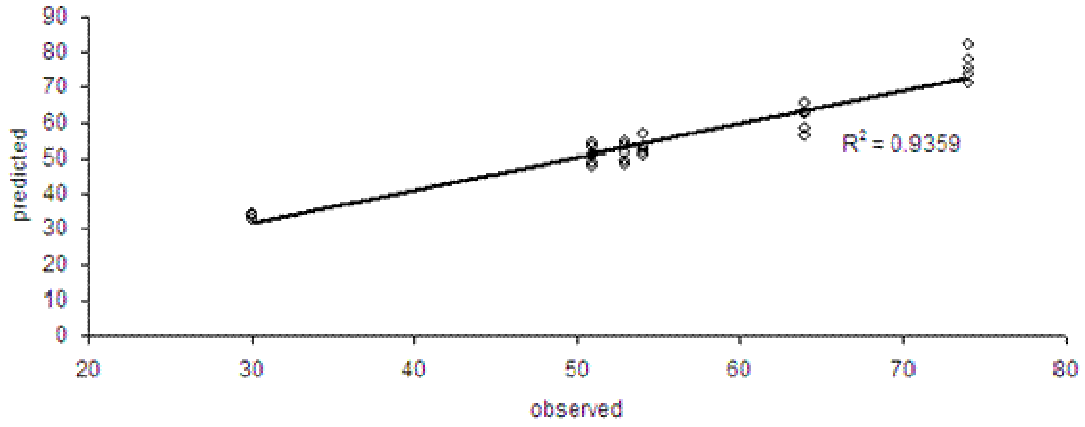


Figure 1 - (a) Scores scatter plot (PC1 *vs.* PC2) and **(b)** Loadings profiles (PC1 and PC2) of the FT-IR spectra of unprocessed, cooked, dry heated, popped, fermented and germinated samples.

With PLS1 calibration it is possible to correlate FT-IR signals with protein digestibility values, it is correct to associate the position of processed sorghum samples in the scores scatter plot (PC1 *vs.* PC2) with the corresponding digestibility value

Using the 1800-1477 cm^{-1} spectral region with standard normal deviates (SNV) pretreatment and 3 latent variables (LV) a calibration model was built with a R^2 of 0.94 and a Root Mean Square Error of Calibration (RMSEC) of 6.27%. The relationship between actual and predicted digestibility values and b vector profile are presented in Figure 2. Extending the spectral region to 1800-750 cm^{-1}, including starch signals, decreased R^2 to 0.53 and increased RMSEC to17.0%, indicating that protein signals are the only responsible for the calibration model.

a)



b)

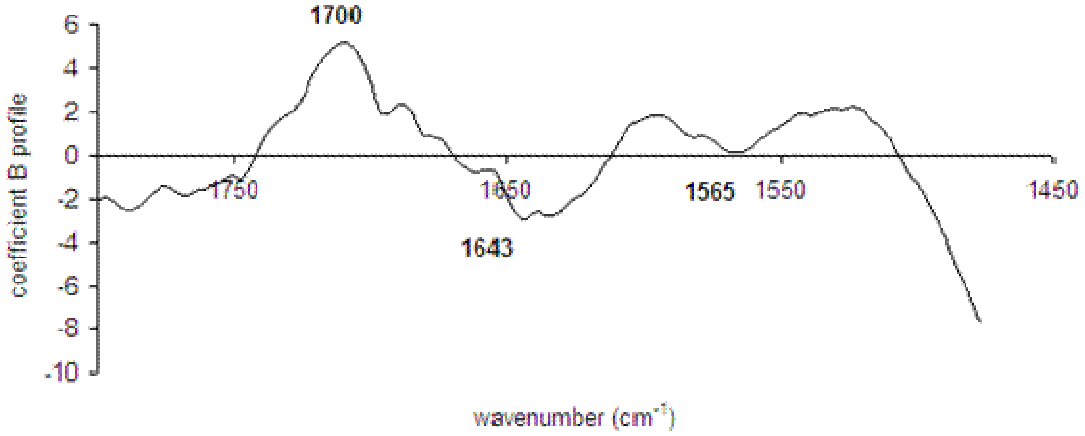


Figure 2 - (a) relationship between actual and predicted digestibility values and **(b)** PLS1 regression ***b*** vector plot of calibration model for digestibility calibration.

3.3. SDS-PAGE results

SDS-PAGE analysis of the samples, shows how different processing methods affected the protein fractions. In the electrophoretic profile of the protein extracted from uncooked flour with *t*-butanol 60%, it is possible to see the presence of high molecular weight aggregates

(HMW), 66 and 45 kDa oligomers and the monomers γ, α1, α2, β and 14kDa. Similar results were obtained by others authors (Duodu et al., 2001; Duodu et al., 2002; Duodu et al., 2003; Nunes et al., 2005) (Figure 3). When the flour was cooked the protein extractability decreased.

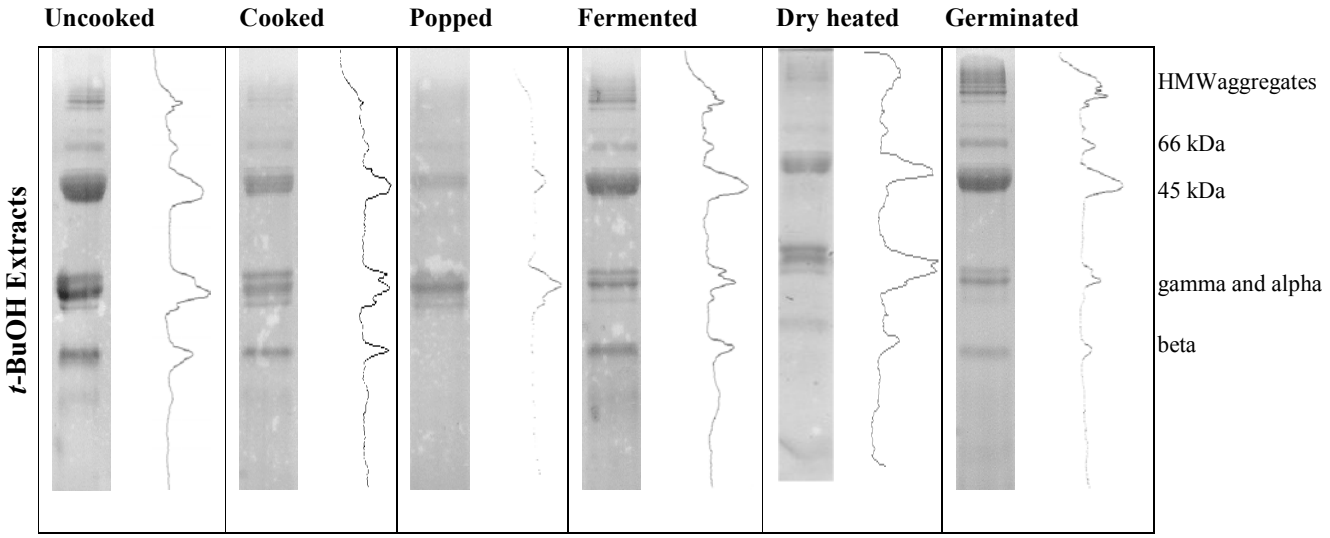


Figure 3 - Electrophoretic gels and electrophoretic profiles of the *t*-butanol prolamin fractions of unprocessed, cooked, popped, fermented, dry heated and germinated samples run under nonreducing conditions.

Table 2 shows that 55.4% of protein remained in the residue after *t*-butanol extraction. Using the same conditions, the bands in the electrophoregram of cooked sample are less intense than the uncooked samples. As it was previously hypothesized (Nunes et al., 2005)**,** the gelatinized starch protects the protein from the solvent extraction. However one cannot discard the effect of the formation of HMW complexes through cooking that decrease protein extractability with *t*-butanol solution (Figure 3). If we take a closer look into the electrophoretic profile of monomer fraction we can easily note that, when compared to cooked sample, the strong decrease is found in α1 and β kafirins. The decrease of γ and α2 kafirins is

less clear (Figure 3). These results suggest that, in this sorghum variety, if there are the formation of HMW aggregates they are mainly formed with α1 and β kafirins.

Table 2 – Amount of protein that remains in the residue after 60% *t*-butanol protein extraction

Samples processing	Protein in residue (% of total protein)
Unprocessing	20.0 ± 0.2 d
Cooking	55.4 ± 0.1 e
Dry heating	19.0 ± 0.1 c
Popping	64.6 ± 0.5 f
Fermenting	15.1 ± 0.3 b
Germinating	12.3 ± 0.2 a

[1] Mean of three replicates.
[2] Means with different letters in same column are significantly different ($p < 0.05$)

As we can also see in Figure 3, when proteins were extracted from popped sorghum the protein extractability was even more affected, when compared to cooked sorghum samples. This decrease is strongly noticed in the monomer fraction. We can observe the disappearance of the β-kafirin from the electrophotetic profile. This effect can be related to the decrease of extractability (t-butanol accessibility to proteins) and/or formation of HMW aggregates. The β-kafirin did not appear after analysis under reducing conditions (Figure 4). When proteins of popped sorghum were extracted using stronger extraction condition (Wallace solution)

(Wallace et al., 1990), there was a slight increase in the protein extraction, however, extractability of uncooked protein was not reached (Figure 4). The residue obtained after protein extraction of popped flour has a considerable amount of protein (64.6%) clearly showing that the extraction was not complete (Table 2).

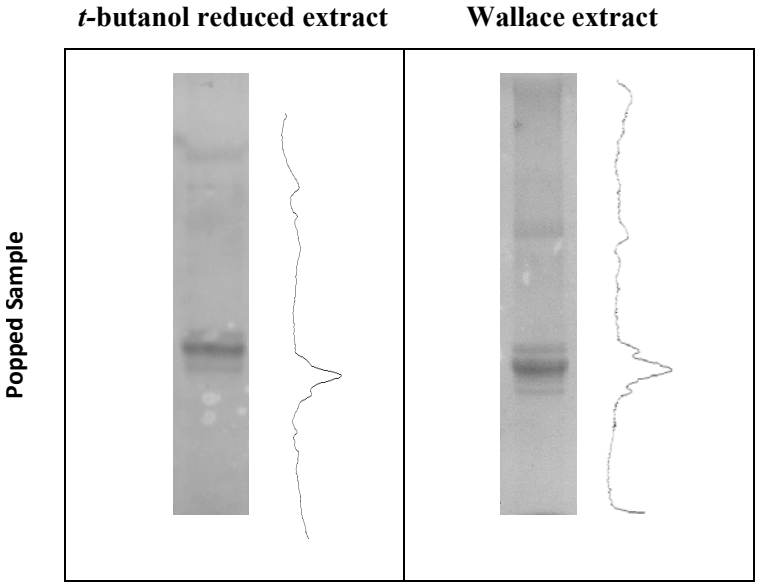


Figure 4 - Electrophoretic gels and electrophoretic profiles of the *t*-butanol and Wallace prolamin fractions of popped samples run under reducing conditions.

The proteins of popped sorghum were less extractable than cooked ones (Table 2), although they were more digestible (Table 1). This phenomenon suggests that protein digestibility is not necessarily related to the extractability behavior of proteins.

According to the thermal treatment developed without the presence of water, we can note that the protein fractions that correspond to the HMW aggregates and 66 kDa oligomer are less intense that in the case of cooking treatment in the presence of water. The other proteins (45 kDa oligomer and γ-, α-, β- monomers) slightly decreased, when compared with uncooked

samples (Figure 3). The amount of protein present in the residue obtained from dry heated flour after *t*-butanol extraction showed that the protein extraction was almost complete (Table 2). Therefore, the HMW aggregates were, in fact, less formed when the flour in dry heated and the protein extractability was not affected because gelatinization did not occur extensively, as it occurred in the case of cooked and popped sorghum. The formation of fewer amounts of HMW aggregates can explain the reason for which protein of dry heated sorghum has a higher digestibility value than for the cooked one.

As it was stated previously, fermented and seven days germinated flour are more digestible than uncooked flour. This can be related to the fact that fermentation and germination decrease the amount of HMW aggregates and oligomers, helping the action of pepsin, enzyme used in the in vitro digestion (Figure 3). At the same time, a decrease of monomers was noticed; those are partially cleaved into small proteic compounds that also help digestion (Figure 3). In both germinated and fermented samples a good protein extraction was noticed, even higher than for uncooked flour (Table 2). This behavior is in some way related to starch attack by endogenous and bacterial enzymes, respectively. Analyzing the monomer fraction it can be noticed that, when compared with uncooked sample, electrophoretic profile of fermented samples shows a strong decrease of $\alpha 1$, $\alpha 2$ and γ and a total disappearance of 14 kDa. This decrease indicates that the monomers were attacked by proteases of fermentative microorganisms. According to germinated sample, all the monomers (γ, $\alpha 1$, $\alpha 2$ and β) decreased, this decrease is more noticed than the fermented samples.

4. Conclusions

Our results confirmed that germination and fermentation are favorable means to improve protein digestibility, by the action of intrinsic and microbial amylases and proteases, respectively. The hydrolysis of starch, by amylases, and of HMW protein fractions, by proteases, increases accessibility of digestive enzymes to sorghum proteins.

Although popping is an explosive process that drastically changes starch structure, it has no consequences in protein digestibility. Changes in protein structure occurs in a slightly extension in popped samples than in cooked ones. In popped samples proteins may suffer denaturation and, consequently, became less extractable. However, digestive enzymes accessibility is not altered.

Dry heated samples present practically the same digestibility values as unprocessed samples and protein extractability was not compromised when this treatment was applied. We can conclude that when water is used in heating process, with concomitant starch gelatinization, protein digestibility value is compromised.

Acknowledgements

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4.1. Análise por microscopia electrónica de varrimento

4.1.1. Materiais e métodos

Preparação das Amostras

As amostras de sorgo foram submetidas ao cozimento em água, banho-maria, pipocagem, fermentação e germinação de acordo com o descrito na secção anterior (secção 3).

Análise por microscopia electrónica de varrimento

A microestrutura das amostras de sorgo submetidas às diferentes formas de processamento foi analisada por microscopia electrónica de varrimento (SEM).

A fixação das amostras ao suporte foi efectuada com fita-cola à base de carbono. De modo a melhorar a condutividade eléctrica durante a observação, foi efectuada a deposição de ouro-paládio por "*Sputtering*" sobre a superfície (JEOL metalizer, FFC-1100, Japan) a 1100-1200 V, 5 mA por 10 minutos. As amostras foram analisadas usando microscópio electrónico de varrimento (Hitachi-S4100, Japão) a 20 kV.

4.1.2. Resultados e discussão

A figura 2 apresenta as imagens de SEM das amostras submetidas às diferentes formas de processamento. Nas micrografias da amostra crua (Figuras 2a e b) é possível visualizar os grânulos de amido embebidos na matriz proteica e rodeados pelos corpos proteicos.

Com o cozimento, verifica-se que os grânulos de amido, bem como os corpos proteicos, são libertados da matriz proteica (Figuras 2c e d). A libertação dos grânulos de amido resulta do processo de gelatinização do amido promovida pelo seu aquecimento em água.

Comparativamente ao cozimento em água, o cozimento em banho-maria parece exercer efeitos menos drásticos no sorgo, verificando-se que as micrografias destas amostras assemelham-se às da amostra crua (Figuras 2e e f). Estes resultados confirmam os obtidos na secção 3, nos quais se verificou a proximidade da amostra crua e da cozida em banho-maria ao longo do eixo PC1 por Análise em Componentes Principais (PCA) dos espectros de FT-IR. Tal como se havia concluído na secção 3, a presença de água durante o tratamento térmico, para além de

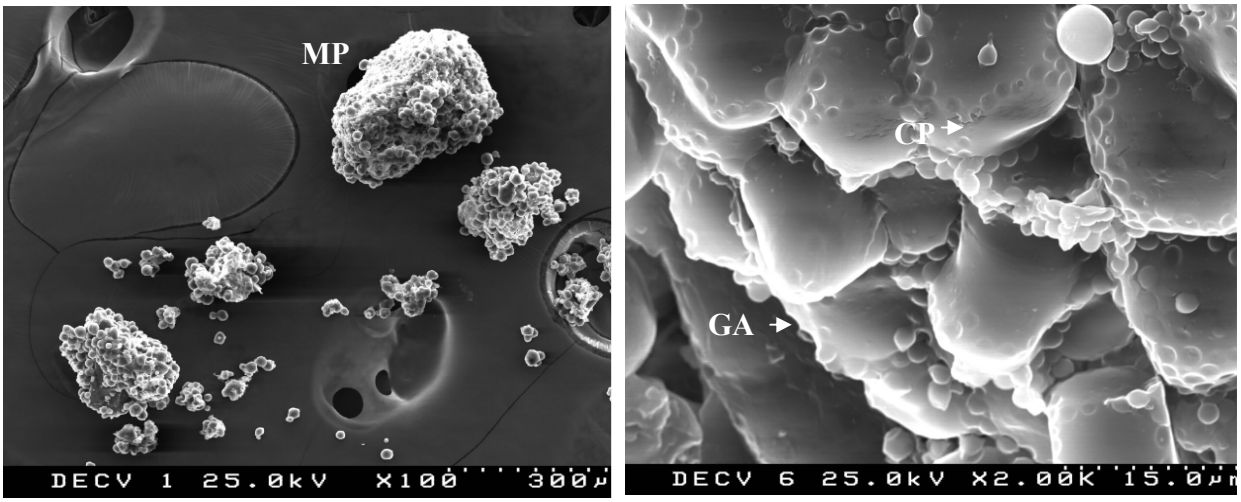
promover a gelatinização do amido, parece exercer alterações nas proteínas, o que poderá estar relacionado com o menor valor de digestibilidade obtido para a amostra cozida em água comparativamente à amostra cozida em banho-maria.

As micrografias das pipocas de sorgo mostram uma perda de integridade das paredes celulares e libertação dos grânulos de amido e de corpos proteicos como resultado do processo explosivo inerente à pipocagem (Figuras 2g e h).

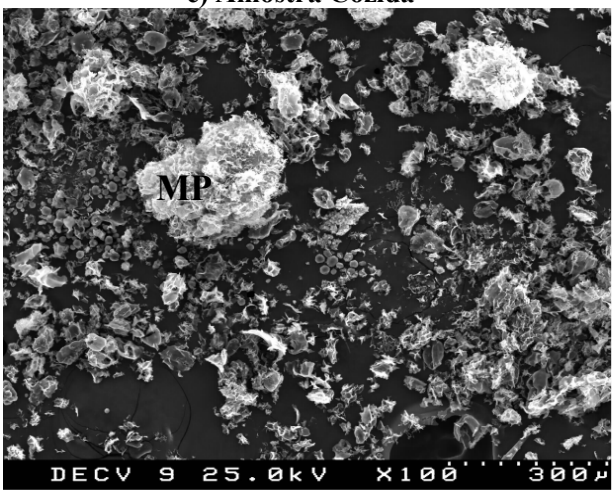
Na amostra fermentada, observa-se um extenso ataque às proteínas e aos grânulos de amido, como resultado da actividade das proteases e amilases microbianas (Figuras 2i e j). O ataque aos corpos proteicos pode ser claramente constatado na figura 2j onde é possível visualizar concavidades na superfície de um grânulo de amido onde os corpos proteicos estavam anteriormente alojados. Na figura 2i verifica-se ainda uma maior dispersão dos grânulos de amido relativamente à matriz proteica e um extenso ataque à mesma.

Efeitos semelhantes podem ser contados na amostra germinada, como resultado de actividade de proteases e amilases endógenas do grão (Figuras 2k e l). Nas figuras 2 k e l evidencia-se, sobretudo, o ataque aos grânulos de amido, que apresentam uma superfície mais irregular e corrompida, comparativamente à amostra crua.

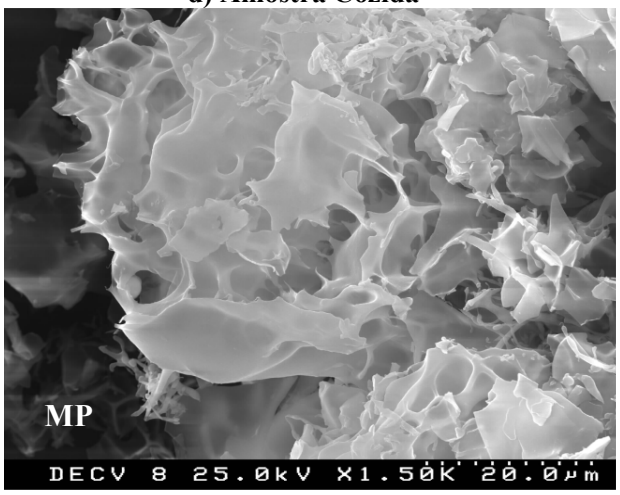
a) Amostra Crua **b) Amostra Crua**



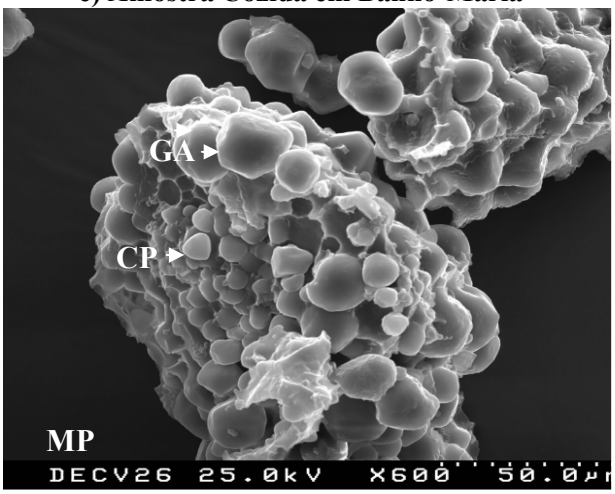
c) Amostra Cozida



d) Amostra Cozida



e) Amostra Cozida em Banho-Maria



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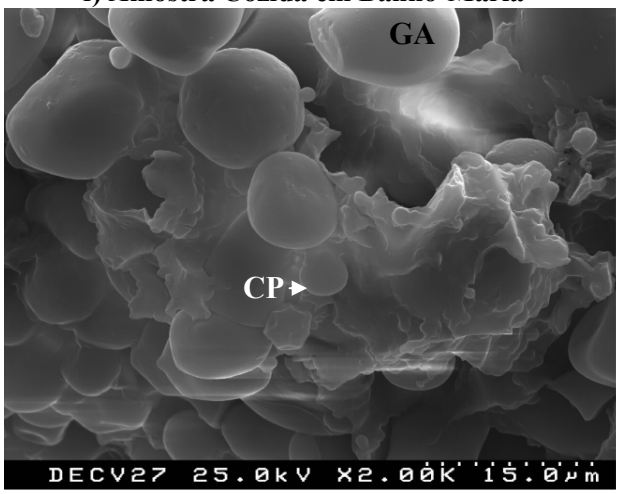
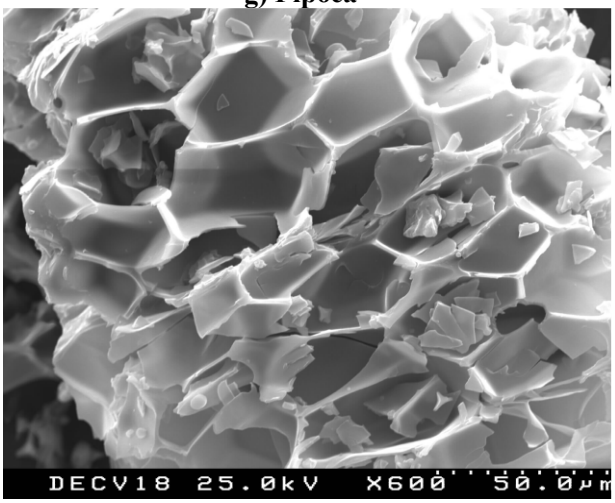


g) Pipoca



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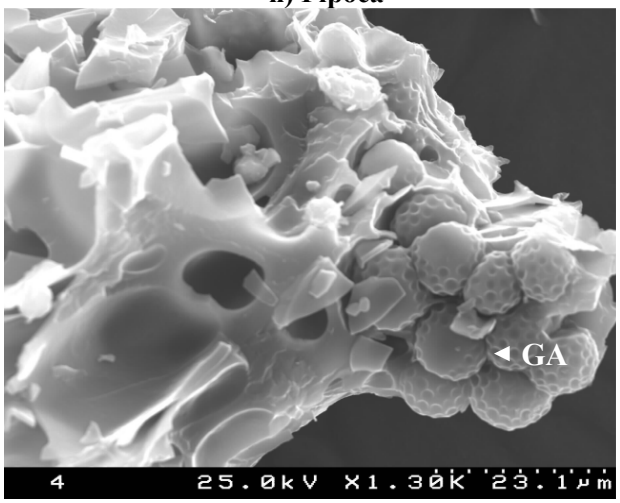
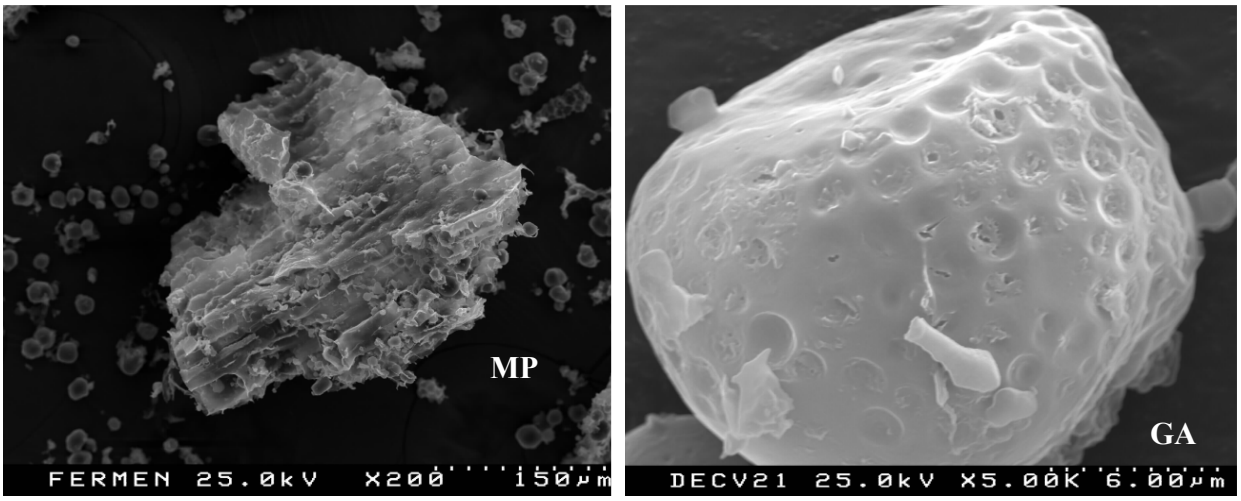


i) Amostra Fermentada

j) Amostra Fermentada



k) Amostra Germinada

l) Amostra Germinada

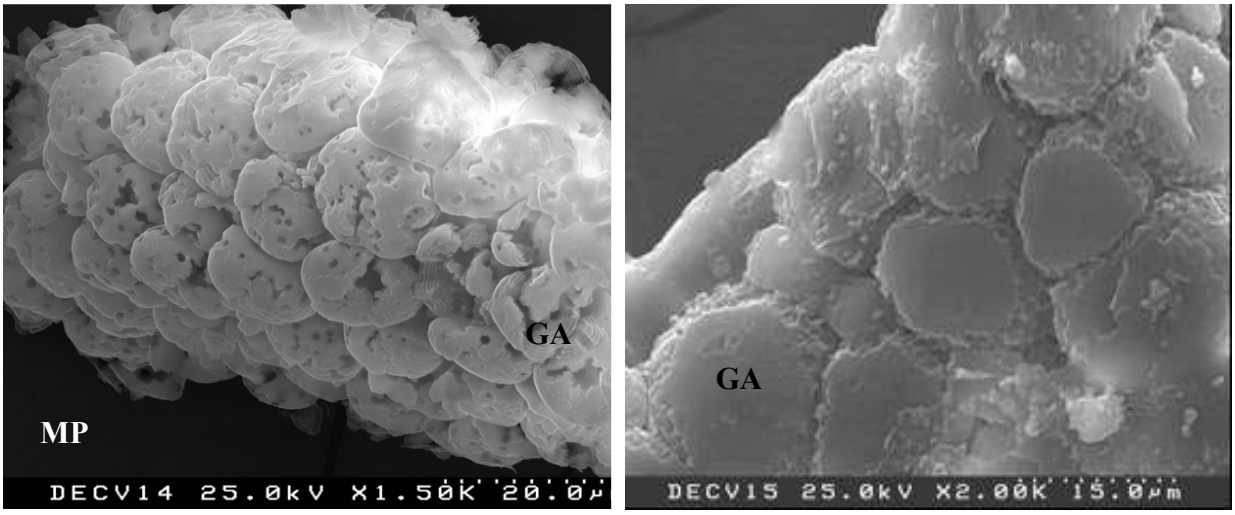


Figura 2 – Micrografias de obtidas por SEM de **a** e **b)** amostra crua, **c** e **d)** amostra cozida, **e** e **f)** amostra cozida em banho-maria, **g** e **h)** pipoca, **i** e **j)** amostra fermentada e **k** e **l)** amostra germinada. GA, grânulo de amido; CP, corpo proteico; MP, matriz proteica.

5. OPTIMIZAÇÃO DAS CONDIÇÕES DE APLICAÇÃO DA TECNOLOGIA DE ALTA PRESSÃO NO INCREMENTO DA DIGESTIBILIDADE PROTEICA DO SORGO

"High Pressure treatments largely avoid/revert decrease of cooked sorghum protein digestibility when applied before/after cooking"

Artigo científico submetido à revista "LWT – Food Science and Technology" (em revisão)"

High pressure treatments largely avoid/revert decrease of cooked sorghum protein digestibility when applied before/after cooking

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ABSTRACT

The results obtained is this work showed that high pressure treatments avoid/revert, to a large extent, cooking deleterious effects that decrease sorghum proteins digestibility, when pressure is applied before/after cooking. The best results were obtained when pressure is applied before the cooking process. Digestibility of cooked sorghum proteins increased from 16.1% to 35.3%/25.4% when pressure at the level of 300 MPa was applied during 15 min before/after cooking, respectively. When 300 MPa were applied for 5 min before cooking, similar results were obtained for digestibility (36.0%).

Analysis of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of sorghum prolamins, revealed that high molecular weight aggregates and a 45 kDa dimer, which usually increase with cooking and are related to protein digestibility decrease, did not significantly change when high pressure is applied.

A relationship between Infra-red (IR) spectra and protein digestibility by means of a Partial Least Square (PLS1) regression was assessed, showing changes in proteins and also on lipids and starch.

It can be concluded that pressurization of sorghum flour, before or after cooking, particularly the former, is a suitable process to greatly improve cooked sorghum protein digestibility.

Key words: *Sorghum bicolor* (L.) Moench, proteins, high pressure, digestibility.

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is one of the most important crops in Africa, Asia and Latin America (Dicko, Gruppen, Traoré, Voragen, & van Berkel, 2006). Sorghum, together with millet, is a major staple food for around 60 million people concentrated in the inland areas of tropical Africa, for who is an important source of proteins (FAO, 2004). A study carried out with urban residents of Polokwane (the capital of the Limpopo province in South Africa), revealed that while sorghum is easily purchasable and widely consumed, increasing consumer demand for sorghum-based products (namely for breakfast meals and for inclusion in infants' cereals used as weaning foods) is occurring (Bichard, Dury, Schonfeldt, Moroka, Motau, & Bricas, 2005).

Among the sorghum proteins, prolamins (known as kafirins) are the most abundant, making up 70-80% of the total endosperm protein. α-kafirin comprises 80% of total kafirins, is located in light staining areas, while β- and γ-kafirins are found in dark staining areas, the former in the interior and the latter inside and at the edge of the protein bodies (Shull, Watterson, & Kirleis, 1992).

Processing methods applied to prepare a wide array of sorghum based foods usually involves cooking (Murty & Kumar, 1995). However, this processing method promotes modifications on the protein fraction, reducing considerably its digestibility and the nutritional value of the foods so prepared (Hamaker, Kirleis, Mertz, & Axtell, 1986; MacLean, Lopez de Romana, Placko, & Graham, 1981). Avoiding or reducing these cooking deleterious effects would be of great importance to produce cooked sorghum based foods, with improved protein nutritional value.

High hydrostatic pressure (HP) is an emerging technology that is being increasingly applied to process foods, being very effective for preservation of foods by pasteurization, as it has little influence on food quality parameters (Castro et al., 2008). HP is also increasingly studied for the modification of functional properties of foods.

Studies on the effects of HP on seed storage proteins were done only on soybean (Alvarez, Ramaswamy, & Ismail, 2008; Puppoa et al., 2008; Wang, Tang, Li, Yang, Li, & Ma, 2008), lupine (Chapleau, & De Lamballerie-Anton, 2003; de Lamballerie-Anton, Delépine, & Chapleau, 2002), and red kidney bean protein isolates (Yin, Tang, Wen, Yang, & Li, 2008) and only few works were done on the effects of HP on protein digestibility (Elgasin, & Kennich, 1980; Klepacka, Porzucek, Piecyk, & Salanski, 1996; Yin et al., 2008). To the best of our knowledge no studies were done on the effect of high pressure on sorghum proteins at all.

The aim of this work was hence to study the effect of HP treatments on cooked sorghum proteins digestibility. Changes on sorghum proteins, evaluated by SDS-PGE and IR, are also reported.

2. Materials and methods

2.1. Sorghum grain

Sorghum bicolor (L.) Moench of Jumbo variety from Australia was purchased in a retail market and stored at 4ºC. Its characterization showed to be a non-tannin (Type I) variety, with 9% of total protein, and 0.32% of polyphenols content.

2.2. Samples preparation

Whole sorghum grains were ground with a coffee mill to pass through a 0.4 mm sieve. Sorghum samples were obtained by mixing 1 part of ground flour with 10 parts of water and cooked samples were boiled in a water bath for 20 min. Cooked and uncooked samples were subjected to HP treatments, freeze-dried, ground, and designated as **Cooked/HP** and **Uncooked/HP**, respectively. To study the effect of HP prior to cooking, a sorghum uncooked sample was submitted to HP, being afterwards cooked, freeze-dried, ground, and designated as **HP/Cooked.**

2.3. High-pressure treatment

Samples were vacuum-conditioned in heat sealed polyethylene bags, using a laboratory scale vacuum packaging machine. High pressure treatments were carried out in a hydrostatic press (Unipress Equipment, Model U33, Poland), with a pressure vessel of 100 mL (35 mm diameter and 100 mm height), surrounded by an external jacket, connected to a thermostatic bath to control the temperature. Prior to the experiments the temperature of the pressure vessel and the samples was equilibrated to 20.0ºC.

High pressure treatments were conducted first at 300 MPa during 15 minutes. To observe the effect of different pressure levels and time under high pressure at 300 MPa, another set of experiments was done at 100 and 450 MPa for 15 min and at 300 MPa during 5 and 30 minutes. Compression was done at a rate of about 200 MPa/min and the temperature inside the vessel increased to a maximum of 23ºC, due to adiabatic heating, when compression ended, but was then kept at 20ºC by the external jacket.

2.4. In vitro protein digestibility determination

All samples were subjected to *in vitro* protein digestion (IVPD) using pepsin (Sigma - P-7000 - 975 U/mg protein) as described by Nunes, Correia, Barros, & Delgadillo (2004). Flour samples (60 mg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 mol/L KH_2PO_4 pH 2 buffer) in a water bath (37°C) for 0 (control sample of IVPD assay) and 120 min. After this period of time, the digestions were stopped by the addition of 100 μL of 2 mol/L NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, room temperature) for 3 min and the supernatants discarded. The residues (with undigested proteins) were washed with 1 mL of 0.1 mol/L K_2HPO_4 pH 7 buffer, centrifuged, washed again with 1 mL of water, and freeze-dried. The content of N of the undigested residues was determined by elementary analysis. The percentage of digested protein, calculated by the difference between the content of total N after 0 and 120 min of digestion, was defined as the *in vitro* protein digestibility value and used as a measure of protein digestibility.

2.5. SDS-PAGE analysis

To study sorghum prolamins profile, 100 mg of sorghum samples were subjected to 24 hour protein extraction with 1.0 mL of *t*-butanol 60%, with mechanical stirring. As the aim of this study was to follow proteins in their non-reduced stage (both polymers and monomers), reducing agents were not used in the extraction. After extraction, the mixtures were centrifuged (2500 x g, room temperature) for 3 min and the supernatants, with extracted proteins, were collected. For SDS-PAGE, 50 μL of *t*-butanol protein extracts were dried under N_2 atmosphere and the obtained residues were dissolved in 20 μL of electrophoretic sample buffer [2% (w/v) SDS, 0.0625 mol/L Tris, 10% (v/v) glycerol, 0.01% (w/v)

bromophenol blue, pH 6.8]. The samples were heated, for 5 min, in a boiling water bath and 7.0 μL were applied in a 15% acrylamide SDS-PAGE gel (Laemmli method). The gels were run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad). Gels were stained with Coomassie Blue R (Pharmacia) and destained with 40% methanol and 10% acetic acid.

2.6. Analysis of SDS-PAGE images

Electrophoretic gels were digitalized without previous drying, in a Hewlett Packard ScanJet 3600C scanner. Each one of the electrophoretic lanes was spited out and separately submitted to a mathematical treatment based on joint density probability estimation (Barros, Safar, Devaux, Robert, Bertrand, & Rutledge, 1997). As result, difference and independence matrices were obtained. The independence matrix corresponds to a noiseless color-coded image. The profile recovered from each image corresponds to its maximum value. All electrophoretic profiles corresponding to electrophoretic bands were submitted to area estimation using the program Origin®, Microcal software Inc., USA. Values presented are expressed as percentage of each protein band area.

2.7. IR analysis

IR spectra were obtained using a Golden Gate single reflection diamond ATR system in a Bruker IFS-55 spectrometer. The spectra were recorded in absorbance mode from 4000 to 500 cm^{-1}, co-adding 128 scans at 8 cm^{-1} resolution. Five replicates were collected for each sample. The obtained spectra were transferred into a data analysis package (Barros, 1999) and for multivariate calibration, IR spectra in the range of 1800-800 cm^{-1} were imported into in-house developed routines for Partial Least Squares regression (PLS1) (Helland, 2001; Martens, 2001; Wold, Sjöström, & Eriksson, 2001)

2.8. Statistical analysis

All results are expressed as means and standard deviation for three replicates, with exception of those obtained by SDS-PAGE analysis. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

When sorghum flour was cooked its protein digestibility was reduced from 42.1 to 16.1 % (Table 1), a value similar to that observed by Nunes et al. (2004) with PAN 8564 sorghum variety. The decrease in sorghum proteins digestibility promoted by cooking, has been attributed to the formation of disulphide cross linkages between β-kafirins and γ-kafirins, located on the surface of sorghum protein bodies (Duodu, Nunes, Delgadillo, & Belton, 2002; Duodu et al., 2002; Emmambux & Taylor, 2009; Wong et al., 2009).

Uncooked sorghum flour treated at 300 MPa of pressure for 15 min showed a protein digestibility value (43.5 %, Tabel 1) similar to that of the uncooked sorghum not submitted to HP. However, the protein digestibility of cooked sorghum, subjected to the same HP treatment before or after cooking, was much higher (Table 1), respectively, 35.3 (1.6-fold) and 25.4% (2.2-fold), compared to the cooked sorghum not HP treated (16.1 %). These results indicate that the 300 MPa HP treatment applied can largely avoid (applied before cooking) or even revert (applied after cooking), the deleterious effect of cooking on sorghum protein digestibility. A slight increase (around more 1.1-fold) of digestibility of extracted kafirins was observed when sorghum was pressure cooked (Emmanbux & Taylor 2009). The higher effect observed when HP was applied prior to cooking can be related to the higher water mobility in

the raw sample during the high pressure treatment. It is known that HP can lead to protein unfolding, due to changes on non-covalent bonds and disruption of SS bonds and to the increase of free SH groups (Messens, van Camp, & Huyghebaert, 1997; Wang et al., 2008).

Table 1 – *In vitro* protein digestibility results of sorghum samples not processed by pressure (uncooked and cooked) and processed by pressure at 300 MPa during 15 min -uncooked (Uncooked/HP), first cooked and then processed (Cooked/HP), and first processed and then cooked (HP/Cooked).

Processed Samples	Digestibility Values (%)[1]
Uncooked	42.1 ± 0.6^{a}
Cooked	16.1 ± 0.7^{b}
Uncooked/HP	43.5 ± 1.0^{a}
HP/cooked	35.3 ± 1.4^{c}
Cooked/HP	25.4 ± 1.4^{d}

[1]Mean of three replicates ± standard deviation. Same letters show non-significant differences ($p > 0.05$)

The enhancement on sorghum flour protein digestibility caused by HP can be a result of changes on non-covalent bonds within protein molecules and disruption of SS bonds and increase of free SH groups, leading to better pepsin accessibility. Another work done with lupine revealed also an increase in protein digestibility of lupine flour samples submitted to HP processing (de Lamballerie-Anton et al., 2002).

In order to try to understand the effect of HP on sorghum proteins that leads to increased protein digestibility, proteins of these samples were extracted and analyzed by SDS-PAGE. The electrophoregram of prolamins from sorghum uncooked flour presented bands that correspond to HMW aggregates, a 66 kDa trimer, a 45 kDa dimer and γ, α, β monomers, with the respective areas presented in Table 2 (similar results were obtained by Nunes et al., 2004).

Table 2 - Quantification of each area of the electrophoretic profiles of sorghum prolamins.

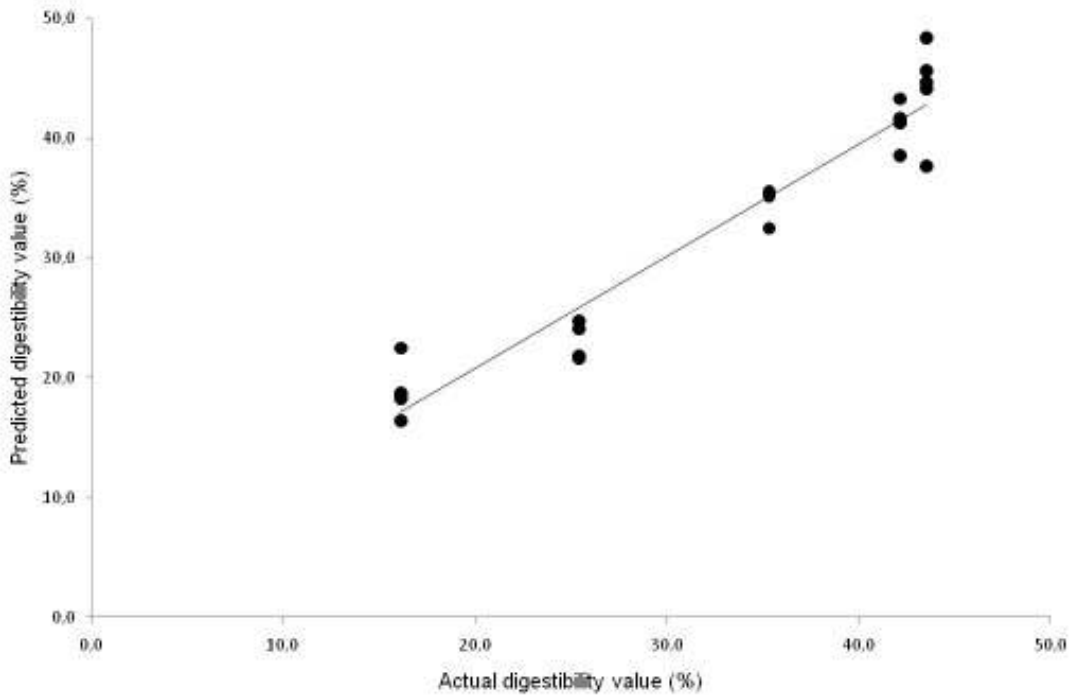
	Uncooked (%)	**Cooked (%)**	**Uncooked/HP (%)**	**HP/Cooked (%)**	**Cooked/HP (%)**
HMW (%)	3.6	6.9	3.4	3.5	3.7
66 (%)	3.7	4.1	2.1	3.0	3.7
45 (%)	34.9	41.9	37.3	36.9	34.3
γ+α (%)	47.0	37.7	45.6	45.0	48.3
β1+β2 (%)	10.8	9.4	11.6	11.6	10.0

Cooked sorghum flour showed an increase in HMW aggregates and 45 kDa dimer areas, a typical behaviour also reported previously by Nunes et al. (2004), which has been associated with the decrease in protein digestibility value caused by cooking. Simultaneously, the disappearance of the β2 monomer, already observed before by Nunes (2004), was detected together with a decrease in bands that correspond to the γ+α monomers (Table 2). These results pointed toward the involvement of the β2 monomer and not the β1 monomer in the formation of HMW aggregates and 45 kDa dimers that increased in prolamin fraction of sorghum after cooking.

No significant differences were observed in the electrophoretic profile areas of uncooked sorghum prolamins, compared to pressure treated uncooked sorghum (Table 2), a result that is

in accordance with the results of protein digestibility. Also, cooked sorghum samples, submitted to high pressure either before or after cooking, showed no remarkable changes in the electrophoretic prolamins profile, compared to uncooked non-pressure treated sorghum flour (a result that is also in agreement with the results of protein digestibility). It is of relevance to highlight the great similarity of the electrophoretic area profiles of these 3 samples. In the presence of high pressure, the HMW aggregates and the 45 kDa dimer, which usually increase with cooking, and the γ+α monomers, that decrease with cooking, did not significantly change when high pressure was additionally applied to cooked sorghum samples (Table 2).

a)



b)

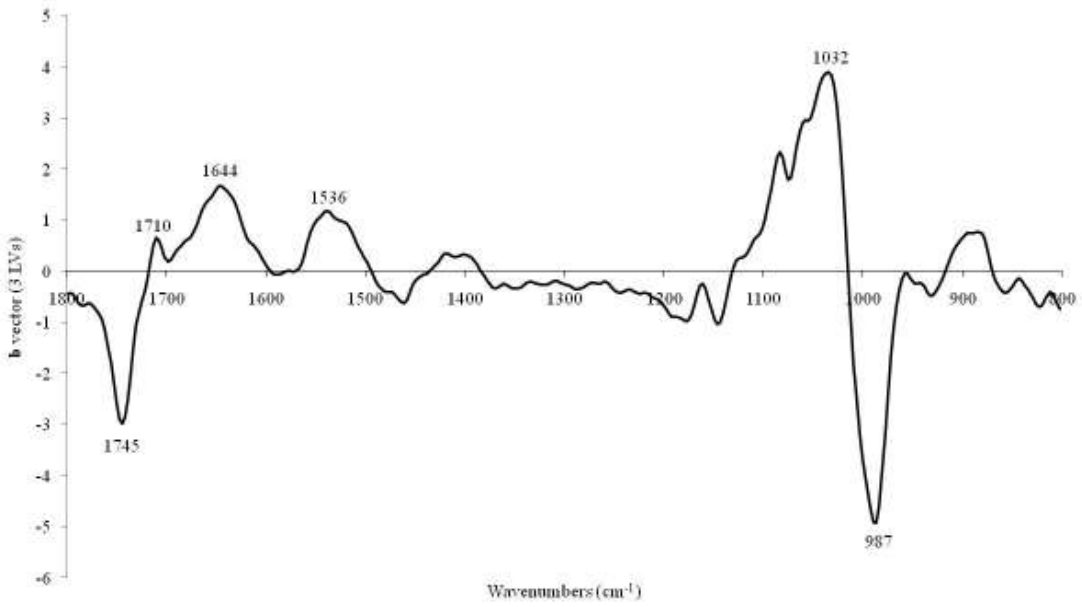


Figure 1 - (a) relationship between actual and predicted digestibility values and **(b)** PLS1 regression **b** vector profile for digestibility prediction.

Using the 1800-800 cm^{-1} spectral region pre-processed with standard normal deviate (SNV), 3 latent variables (LV) were found to give a calibration model with predictive power. The cross-validated coefficient of determination (Q^2) was found to be 0.98, the Root Mean Square Error of Cross-Validation (RMSECV) was 1.2 %, and the Root Mean Square Error of Prediction (RMSEP) was 9.9%. The relationships between actual and predicted digestibility values, as well as, the corresponding **b** vector profile are plotted in Figure 1. The **b** vector profile (Figure 1b) showed that the increase in digestibility values of samples were positively related to proteins characteristics bands (1644 and 1536 cm^{-1}). This supports the findings observed by SDS-PAGE that high pressure promotes changes on proteins leading to an increase in protein digestibility. It was also noticed a shift from a triglyceride characteristic peak, at 1743 cm^{-1}, to a free fatty acids peak at 1710 cm^{-1} and a shift from the maximum of

starch characteristic peak from 1032 to 987 cm^{-1}. Several authors reported that high pressure can promote gelatinization of starch granules at room temperature (Blaszczak, Fornal, Kiseleva, Yuryev, Sergeev, & Sadowska, 2007; Buckow, Heinz, & Knorr, 2007).

Since the most beneficial effect of the 300 MPa pressure treatment on sorghum protein digestibility was observed when HP was applied before cooking, the influence of different pressure levels (100 and 450 MPa during 15 min) and HP treatment times (300 MPa during 5 and 30 min), was studied by application to samples before cooking, on a second set of experiments.

Table 3 – *In vitro* protein digestibility results of cooked sorghum samples submitted to HP treatment, at different pressure levels and times, before cooking.

Processed Samples	Digestibility Values (%)[1]
100 MPa, 15 min	27.6 ± 1.3^{b}
450 MPa, 15 min	24.3 ± 0.8^{d}
300 MPa, 5 min	36.0 ± 0.5^{c}
300 MPa, 30 min	28.7 ± 1.1^{b}

[1]Mean of three replicates ± standard deviation. Same letters show non-significant differences ($p > 0.05$)

In all cases, a significant increase of protein digestibility was achieved when compared to the cooked sample without HP treatment (16.1 %), with values ranging from a 27.6% (450 MPa, 15 min) to 36.0% (300 MPa, 5 min). When samples were submitted to a lower pressure (100 MPa), the high pressure treatment was less efficient on avoiding the decrease of protein

digestibility caused by cooking, resulting on a lower digestibility value (27.6%). A similar result was observed when a higher pressure (450 MPa) was applied, resulting in a digestibility value of 24.3%. A possible explanation for these results, supported by conclusions of other authors (Wang et al., 2008; Yin et al., 2008), is that up to certain levels of pressure, protein unfolding and disruption of SS bonds occur (which may be responsible to a higher accessibility of pepsin to prolamins). However, at higher pressure levels, a subsequent aggregation/re-association of unfolded proteins takes place due to hydrophobic interactions, thus decreasing protein digestibility. The same negative effect seems to occur when duration time of HP treatment is increase to 30 min.

4. Conclusions

The results obtained in this work indicate that high pressure treatments, applied before/after cooking of sorghum flour, largely avoid/revert deleterious effects promoted by cooking on sorghum proteins digestibility. It was found that pressurization of sorghum flour at 300 MPa during 5 and 15 min before cooking gives the best results, yielding cooked sorghum samples with protein digestibility values of, respectively, 36.0 % and 35.3 % that compare with a value of 16.1 % for cooked sorghum not submitted to pressure and 42.1 % for uncooked sorghum. In accordance, electrophoretic sorghum protein profiles, of the pressure-treated samples were found to be similar to that of uncooked sorghum. By PLS1 regression it was possible to related IR signals with protein digestibility values and to associate proteins digestibility improvement with changes on proteins, while changes on lipids and starch caused by the pressure treatments were also detected. The results of this work indicate pressure treatments of sorghum flour as a suitable way to greatly improve cooked sorghum protein digestibility.

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6. ACOMPANHAMENTO DO PROCESSO GERMINATIVO DO SORGO AO LONGO DO TEMPO

"Protein profile and malt activity during sorghum germination"

Artigo científico publicado na revista "Journal of the Science of Food and Agriculture"

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Protein profile and malt activity during sorghum germination

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Abstract

BACKGROUND: The effect of germination time on major sorghum macromolecules was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Fourier transform infrared (FTIR) spectroscopy and scanning electron microscopy. Sorghum flour was also incubated with sorghum malt to evaluate amylase and protease activities.

RESULTS: The SDS-PAGE results showed that levels of high-molecular-weight aggregates, β monomer and γ and α monomers increased by 120.5, 20.3 and 12.7% respectively during the first few days of germination. This behaviour shows the enhancement of prolamin extractability and concentration in sorghum as a result of starch degradation. After the third day, proteins suffered degradation by intrinsic proteases. FTIR spectroscopy showed a gradual decrease in lipid and protein levels and starch structural changes during the germination period. These modifications are due to intrinsic lipases, proteases and amylases. Sorghum flour was incubated with different malt samples resulting from 0–7 days of germination. It was found that a 5 day malt led to a sixfold increase in carbohydrate digestibility and a fourfold increase in free amino acid content.

CONCLUSION:A 5 day sorghum malt is the best inoculum for porridge preparation, representing an inexpensive, effortless and culturally acceptable way to prepare weaning foods at domestic and industrial levels.

Keywords: prolamins; weaning food; FTIR; SDS-PAGE

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench), one of the five most important cereals in the world's food supply, is a major food crop in semi-arid regions of Africa and Asia.[1] It represents an important source of energy, proteins, minerals and vitamins for the inhabitants of underdeveloped countries.[2] Sorghum is consumed in different traditional forms in various geographical areas.[3] However, its poor nutritional quality, attributed to its low starch availability,[4] low protein digestibility,[5] deficiency of essential amino acids[6] and presence of antinutritional factors such as tannins,[7] has deleterious effects on the nutritional condition of the population.

In developing countries, weaning with sorghum products has been associated with undernourishment in human infants. Sorghum weaning foods have low energy and nutrient density and are a major cause of malnutrition among 6–24-month-old children in sub-Saharan Africa.[8] As sorghum porridges are very thick, they are diluted to a flour concentration of 5–10% to attain a viscosity of less than 3000 cP in order to facilitate swallowing. However, this concentration provides them with an energy density of only 0.2 kcal g^{-1}, which is below the recommended 0.75 kcal g^{-1} provided by breast milk and too low to meet infant energy requirements.[9–12]

An important characteristic of many sorghum-based foods is that they have undergone a lactic acid process and/or a malting process during their production. These technologies improve the nutritional and functional properties of sorghum.[13]

Germination is a common practice in sorghum-producing areas. Grains are malted for the production of weaning foods, opaque beers and other traditional dishes. Germination triggers the enzyme system of sprouting seeds, leading to the breakdown of proteins, carbohydrates and lipids into simpler forms.[14] This processing method activates intrinsic amylases, proteases, phytases and fibre-degrading enzymes that disrupt protein bodies, thereby increasing nutrient accessibility.[15]

The use of malt in porridge making is referred to as 'power flour' or 'amylase-rich flour' (ARF) technology.[13] ARF technology is of particular importance in weaning foods. The addition of small quantities of ARF to freshly prepared thick gruels liquefies them through the action of α-amylase. The porridge viscosity is thus reduced without lowering its nutrient and energy density.[16] In addition, porridges made with malted grain have greatly improved carbohydrate

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digestibility and palatability.[13] Cereal malts are also used to initiate spontaneous fermentation in a number of indigenous African foods.[17,18] Malt addition to sorghum flour prior to fermentation results in the production of free amino acids and small peptides required for micro-organism nutrition during fermentation.

The aim of the present study was to investigate the simultaneous changes that take place in sorghum macromolecules at different stages of the germination process. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to evaluate changes in prolamin electrophoretic profiles. Fourier transform infrared (FTIR) spectroscopy in tandem with multivariate analysis was used to assess modifications in sorghum grain chemical composition. The microstructure of 0 and 7 day germinated samples was analysed by scanning electron microscopy (SEM) to observe changes in starch granules and protein bodies. Sorghum flour was also incubated with sorghum malt resulting from different days of germination. With this study we can evaluate optimal conditions for using malt prior to fermentation in order to improve sorghum weaning food quality.

EXPERIMENTAL

Sorghum grain

Sorghum bicolor (L.) Moench of the Australian variety Jumbo was purchased from a retail market and stored at 4 °C.

Sorghum germination

Before germination, sorghum grains were cleaned manually with water, 700 mL L^{-1} ethanol and hypochlorite solution containing 10 mL L^{-1} available chlorine, then washed several times with sterilised water. This treatment was applied to prevent the growth of moulds. The washed grains were soaked in sterilised water for 40 h and subsequently drained and spread over paper filters on pre-sterilised trays. Germination was carried out in an incubator at 26 °C for 7 days, revolving the malt daily (to prevent excessive root malting) and spraying twice a day with sterilised water (to prevent drying-out). Aliquots (100 g) were taken daily for SDS-PAGE and FTIR analyses and for use as inocula for sorghum flour. All samples were dried in a vacuum oven and derooted. Finally, sorghum grains were ground in a coffee mill and sieved to obtain sorghum flour of 0.4 mm granularity.

SDS-PAGE analysis

To study the prolamin profiles of samples from 1–7 days of germination, 100 mg of each germinated sample was submitted to 1 h of protein extraction with 0.5 mL of 600 g L^{-1} *tert*-butanol under mechanical stirring. As the aim of this study was to follow proteins in their non-reduced state (both polymers and monomers) during germination, reducing agents were not used in the extraction. After extraction the mixtures were centrifuged at 2500 × *g* for 3 min at room temperature. Supernatants with extracted proteins were obtained. For SDS-PAGE, 20 μL of each *tert*-butanol protein extract was dried under nitrogen and the obtained residue was dissolved in electrophoretic sample buffer containing 20 gL^{-1} SDS, 0.0625 mol L^{-1} Tris, 100 mL L^{-1} glycerol and 0.1 gL^{-1} bromophenol blue (pH 6.8). To observe the oligomers, non-reducing conditions were applied. When reducing conditions were needed, 50 g L^{-1} 2-mercaptoethanol (2-ME) was included in the sample buffer. Samples were heated for 5 min in a boiling water bath and 7 μL aliquots were applied to 150 g L^{-1} SDS-PAGE acrylamide gels (Laemmli method). The gels were run in a Mini-Protean II electrophoretic cell equipped with a Power Pac 300 (Bio-Rad, Hercules, USA). Electrophoresis was conducted at 170 V for 1.5 h until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Gels were stained with Coomassie Blue R (GE Healthcare, Uppsala, Sweden) and destained with 400 mL L^{-1} methanol and 100 mL L^{-1} acetic acid.[19]

Analysis of SDS-PAGE images

Electrophoretic gels, without previous drying, were digitised in a Hewlett Packard ScanJet 3600C scanner (California, USA). Each acquired image was converted into a matrix representing the different colour intensities (colour-coded images).[20] Individual electrophoretic lanes were submitted to a mathematical treatment based on joint density probability estimation.[21] As a result, difference and independence matrices were obtained. The independence matrix corresponds to a noiseless colour-coded image. The profile recovered from each image corresponds to its maximum value. This mathematical treatment acts as a filter that enhances protein concentration determination.

All electrophoretic profile regions corresponding to electrophoretic spots were submitted to area estimation using the program Origin (Microcal Software Inc., Northampton, Massachussets (MA), USA).[20] The obtained normalised areas were expressed in arbitrary units (a.u.).

FTIR analysis

FTIR spectroscopy was used to evaluate the effects of germination on the samples. FTIR spectra were obtained using a Golden Gate single reflection diamond attenuated total reflection (ATR) system in a Bruker IFS-55 spectrometer (Ettlingen, Germany). The spectra were recorded in absorbance mode from 4000 to 500 cm^{-1}, co-adding 128 scans at 8 cm^{-1} resolution. Five replicates were collected for each sample. The obtained spectra were transferred into a data analysis package.[22] The 1780–800 cm^{-1} region was selected for principal component analysis (PCA),[23] because it comprises signals from lipids (1743 cm^{-1}), proteins (1650–1500 cm^{-1}) and starches (~1000 cm^{-1}), major components of

sorghum flour. Each spectrum was standard normal variate (SNV) corrected.

SEM analysis

The microstructure of 0 and 7 day germinated samples was observed with a Hitachi S4100 scanning electron microscope (Tokyo, Japan) at 20 kV. Samples were fixed with carbon adhesive on steel supports and coated with gold/palladium using a JEOL FFC-1100 metalliser (Tokyo, Japan) at 1100–1200 V and 5 mA for 10 min.

Incubation of sorghum flour with malt samples

Sorghum grains were ground in a coffee mill and sieved to obtain sorghum flour of 0.4 mm granularity. Eight samples of 1 g of sorghum flour in 10 mL of distilled were placed in a boiling water bath for 15 min to promote starch gelatinisation. After cooking, the resulting porridges were placed in a water bath to cool to 35 °C. Each cooled porridge was incubated with 0.5 g of a sorghum malt sample and 5 mL of distilled water. Different malt samples resulting from 0–7 days of germination were used as inocula for the eight porridges. The mixtures were left to stand for 30 min at room temperature. At the end of the incubation period, samples were boiled for 5 min. After centrifugation at 2500 × *g* for 3 min at room temperature the supernatants were analysed for total sugars and amino acids as a measure of protease and amylase activities.

Determination of total soluble sugars

A modified phenol/sulphuric acid method was used to determine the total sugars present in the sample supernatants.[24] First 1 mL of 50 mL L^{-1} phenol was added to 100 μL of sample. Then 1mL of concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 10 min. After cooling to room temperature (~25 °C) in a cold water bath, the absorbance at 490 nm was measured using a Shimadzu UV-160A spectrophotometer (Tokyo, Japan). The concentration of sugars was determined by reference to a standard curve previously prepared with glucose. Blanks were prepared by substituting distilled water for sample solution. All measurements were made in triplicate.

Determination of free amino acids

The quantitative measurement of free amino acids in the sample supernatants was made using the ninhydrin reaction.[25] First 2 mL of buffered ninhydrin reagent (0.8 g of ninhydrin and 0.12 g of hydrindantin dissolved in 30mL of 2-methoxyethanol plus 10 mL of 4 molL^{-1} acetate buffer, pH 5.5) was added to 2 mL of sample and heated in a boiling water bath for 15 min. The mixture was then cooled to room temperature (~25 °C) and 3 mL of 500 mL L^{-1} ethanol was added. After 10 min the absorbance at 570 nm was measured using a Shimadzu UV-160A spectrophotometer. The concentration of amino acids was determined by reference to a standard curve previously prepared with arginine. Blanks were prepared by substituting distilled water for sample solution. All measurements were made in triplicate.

Statistical analysis

All values are expressed as mean ± standard deviation of three replicates. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

SDS-PAGE analysis

Figure 1 shows a typical non-reduced kafirin electrophoretic profile with spots characteristic of high-molecular-weight (HMW) aggregates, 66 and 45 kDa oligomers, γ and α monomers and β monomer. Since the dimension and colour intensity of an electrophoretic spot are considered to reflect the amount of protein contained in it, by quantifying the electrophoretic spot areas it is possible to study variations in the amount of kafirins during the 7 days of germination.

Figures 1 and 2 show that the HMW aggregate concentration increased from day 1 (8.3 a.u.) to day 2 (16.7 a.u.) of germination. After another small increase from day 2 to day 3 (18.3 a.u.), no further changes were observed up to day 7 (18.2 a.u.).

The 66 kDa oligomer concentration showed no significant variation during the germination period, remaining at ~4.0 a.u.

The 45 kDa oligomer concentration remained constant during the first 3 days of germination (~38.0 a.u.). Thereafter it decreased slightly, reaching 33.0 a.u. at day 7.

The γ and α monomer concentration increased from 24.5 to 27.6 a.u. during the first 2 days

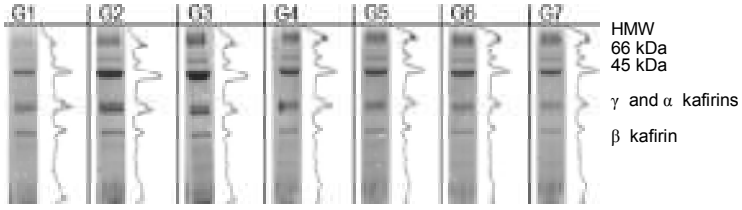

Figure 1. Electrophoretic profiles of constituents of kafirin fractions extracted from germinated samples and analysed under non-reducing conditions.

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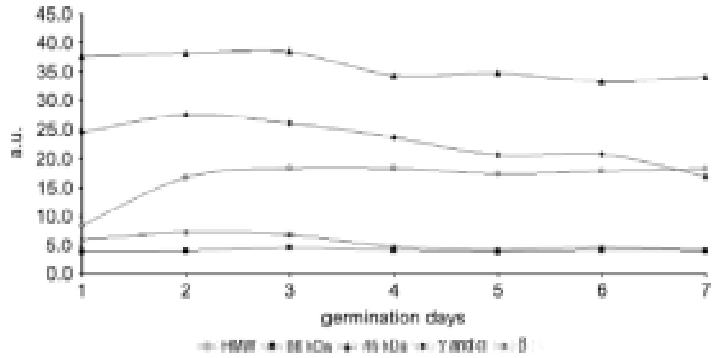

Figure 2. Electrophoretic normalised areas (a.u.) of each constituent of kafirin fractions extracted from germinated samples and analysed under non-reducing conditions, plotted as a function of germination days.

of germination. Thereafter it decreased gradually, reaching 16.9 a.u. at day 7.

The β monomer concentration increased slightly from day 1 (5.9 a.u.) to day 2 (7.1 a.u.), then decreased until reaching 4.0 a.u. at day 7.

With the exception of the 66 kDa oligomer, whose concentration did not change significantly throughout the 7 days of germination, all other electrophoretic spot areas either increased or did not change during the first few days of germination and then decreased up to day 7 of germination. α-Amylase activity during malting results in modifications in grain starch.[13] These starch modifications lead to an enhancement of prolamin extractability, which may be related to the increase in electrophoretic spot areas observed during the first few days of germination. In addition, carbohydrate respiration can also promote prolamin concentration in malted sorghum. The observed decrease in prolamin electrophoretic spot areas after 2 or 3 days of germination is due to prolamin hydrolysis. Other studies indicated that malting results in the production of proteolytic enzymes that hydrolyse a proportion of prolamins and glutelins into simpler forms.[26,27]

When the kafirin extracts were analysed under reducing conditions, the electrophoretic profiles underwent some visible changes: disappearance of the HMW aggregate and 66 kDa oligomer electrophoretic spots, strong decreases in the 45 kDa oligomer electrophoretic spot and concomitant increases in the γ, α and β monomer electrophoretic spots (Fig. 3). Reducing conditions cause S–S bond cleavage, which leads to disruption of the oligomers and the appearance of their constituents.

Figure 3 also shows the appearance of a new band corresponding to a 14 kDa monomer. This could indicate that the sorghum variety studied contains an S–S-bonding monomer that is released when reducing conditions are applied.

Figure 4 shows the variation in electrophoretic spot areas of (a) the γ and α monomers and (b) the 45 kDa oligomer, β monomer and 14 kDa monomer under reducing conditions as a function of germination period. The γ and α monomer concentration increased from 63.0 to 78.6 a.u. during the first 3 days of germination, then decreased until reaching 66.4 a.u. at day 7 (Fig. 4(a)). The 45 kDa oligomer concentration decreased from 1.4 to 0.9 a.u. during the first 2 days, decreased further to 0.5 a.u. from day 3 to day 4 and then remained almost constant until the end of the germination period. The β monomer showed a similar behaviour to the γ and α monomers: an increase in concentration from 2.1 to 2.5 a.u. during the first 3 days and then a slight decrease until reaching 1.0 a.u. at day 7. The 14 kDa monomer profile was similar to that of the β monomer: an increase in concentration from 3.4 to 4.3 a.u. during the first 3 days, followed by a decrease to 3.3 a.u. at day 7 (Fig. 4(b)).

These profile behaviours are again attributable to the enhancement of the extractability and concentration of prolamins as a result of starch degradation, followed by their degradation by intrinsic proteases.

FTIR and SEM analyses

PCA of the FTIR spectra in the 1780–800 cm^{-1} region showed a clear trend of the germinated samples

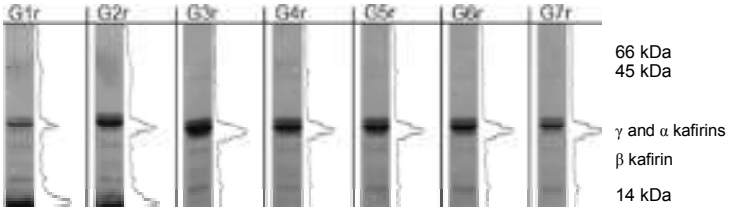

Figure 3. Electrophoretic profiles of constituents of kafirin fractions extracted from germinated samples and analysed under reducing conditions.

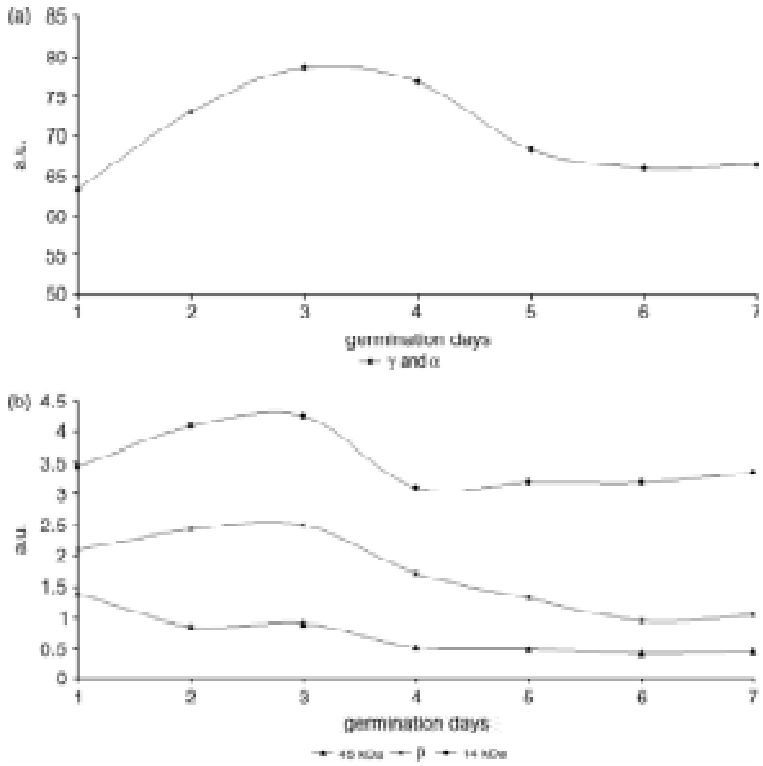

Figure 4. Electrophoretic normalised areas (a.u.) of (a) *γ*- and *α*-prolamin and (b) 45 kDa oligomer, 14 kDa monomer and *β*-prolamin extracted from germinated samples and analysed under reducing conditions, plotted as a function of germination days.

along the PC1 axis (Fig. 5(a)). The sample distribution indicates gradual modifications in sorghum constituents during the germination period. With increasing germination time the samples are distributed from PC1(−) (1 and 2 day germinated samples) to PC1(+) (5–7-day germinated samples). The loading profile (Fig. 5(b)) shows that samples located at PC1(−) are characterised by signals from lipids (1743 cm^{-1}) and proteins (1639 and 1532 cm^{-1}). The occurrence of these signals indicates a continual decrease in lipid and protein levels with increasing germination time as a result of intrinsic lipase and protease activities. Samples located at PC1(+) are mainly characterised by signals from carbohydrates (995 cms^{-1}) that can be ascribed to starch structural changes as a result of amylase activity. The SEM images of a 7 day germinated sample (Figs 6(b) and 6(c)) confirm a strong attack on starch granules and protein bodies in comparison with a non-germinated sample (Fig. 6(a)). In the non-germinated sample it is possible to see intact starch granules surrounded by protein bodies. Moreover, the protein bodies in the germinated grains become detached from the starch granules and are no longer visible. The starch granules appear eroded in the places where the protein bodies were located. These findings are consistent with the FTIR results that show a decrease in protein content and changes in starch molecules.

Incubation of sorghum flour with malt samples

The various germinated samples containing different enzymatic systems were used to incubate sorghum flour in order to evaluate their action on major sorghum biopolymers. Changes in total soluble sugars and free amino acids of samples submitted to incubation with 0–7 day germinated malts are presented in Table 1.

It can be seen that the concentration of soluble sugars increased with increasing germination time

Table 1. Total soluble sugars and free amino acids of sorghum flour incubated with different malt samples resulting from 0–7 days of germination

Malt sample	Total soluble sugars (mg L^{-1})	Free amino acids (mg L^{-1})
0 day germinated	579.5 ± 21.5a	280.9 ± 99.5a
1 day germinated	2382.8 ± 28.6b	445.2 ± 29.2b
2 day germinated	3550.1 ± 4.1c	590.7 ± 122.1b
3 day germinated	3861.0 ± 89.8d	981.7 ± 52.6c
4 day germinated	4037.7 ± 40.2e	1215.6 ± 81.1d
5 day germinated	4174.3 ± 73.4f	1442.2 ± 114.9e
6 day germinated	4588.9 ± 8.2g	1266.9 ± 56.5e
7 day germinated	4676.1 ± 78.2g	1240.3 ± 77.6e

Values are mean ± standard deviation of three replicates. Means followed by the same letter within a column are not significantly different ($P > 0.05$).

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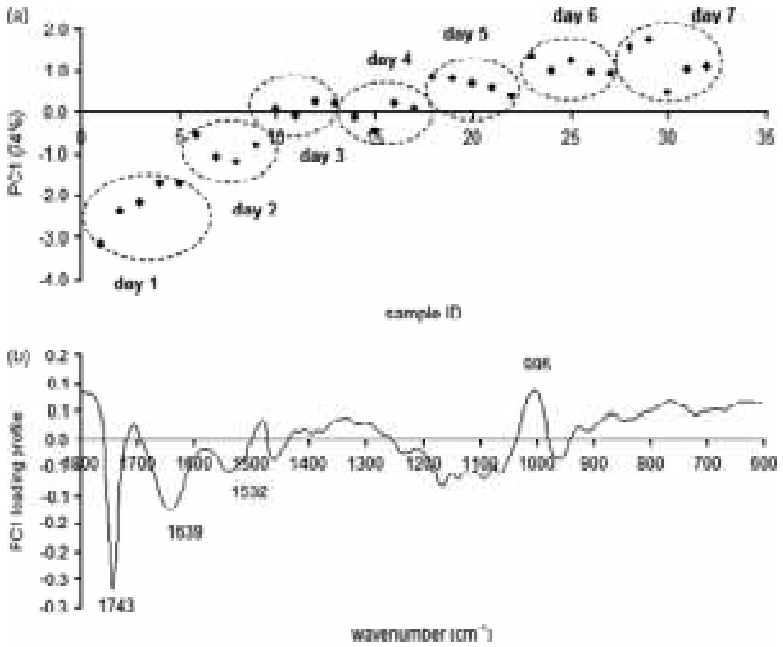

Figure 5. (a) PCA score plot (PC1 as a function of sample identification) and (b) PCA loading plot profile (PC1) of FTIR spectra of germinated samples.

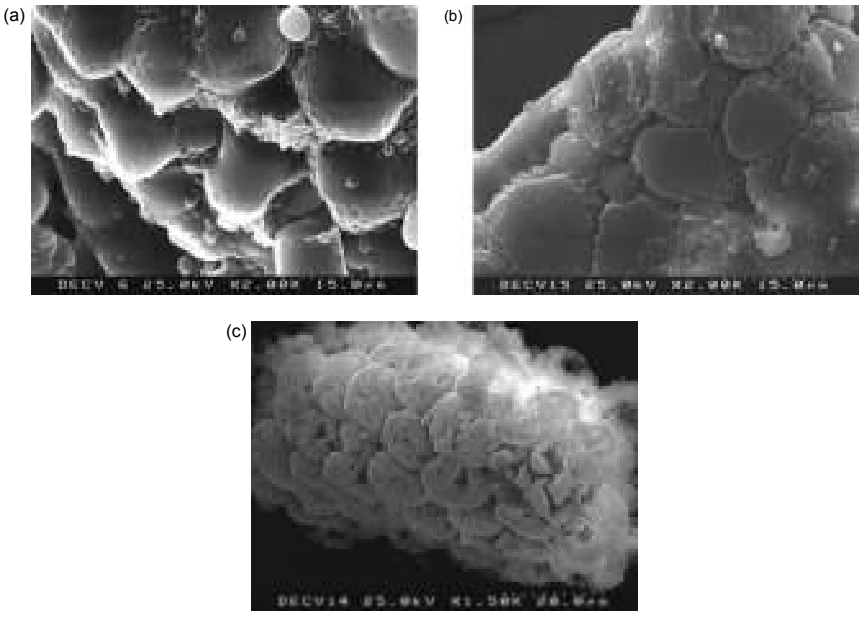

Figure 6. SEM images of (a) non-germinated and (b, c) 7 day germinated sorghum flour.

of the malt used as inoculum. A sevenfold increase was observed from 0 day malt to 7 day malt. Other researchers have also reported an increase in sugar levels with increasing germination time.[28 – 30] The increase in soluble sugars is correlated with the amylase activity in germinating grains.

Table 1 shows a successive increase in amino acid levels when samples were incubated with inocula resulting from 0 days (280.9 mg mL^{-1}) to 5 days (1442.2 mg mL^{-1}) of germination. This increase is due to protein hydrolysis by the action of intrinsic proteases. When samples were incubated with malt

germinated for longer than 5 days, there was a 12.2% decline in free amino acids. These findings are in agreement with those of Mugula *et al*.,[31] who attributed this decrease to the migration of nitrogen compounds to the roots and shoots. The concentration of amino acids is dependent on the balance between their release by degradation of proteins into peptides and amino acids and their removal to new tissues for plant structure and enzyme synthesis.[26,31]

CONCLUSIONS

In this study a combination of SDS-PAGE, FTIR spectroscopy and SEM allowed the analysis of successive changes occurring throughout 7 days of sorghum germination. During the first 3 days of germination it was found that prolamins were more accessible and consequently more extractable as a result of starch hydrolysis by intrinsic amylases. Thereafter, prolamins were hydrolysed by proteases into peptides and amino acids. After day 5 of germination the migration of nitrogen compounds to the roots and shoots was confirmed.

Germination causes activation of intrinsic amylases and proteases that disrupt starch granules and protein bodies into simpler forms. As a result, malt samples contain free sugars and amino acids released during germination. When sorghum flour is incubated with these malts, it becomes enriched with soluble sugars and amino acids. On the other hand, malt samples also contain amylases and proteases. These enzymes lead to the breakdown of sorghum flour starches and proteins and the release of additional soluble sugars and amino acids. This is of paramount importance in weaning foods, as porridge viscosity could be reduced and carbohydrate and protein digestibility thus improved.

The findings of this study suggest that utilisation of 5 day germinated sorghum flour in weaning food preparation leads to an enhancement of the nutritional properties of the product. This method of sorghum processing could be useful in countries where the food intake of young children consists mainly of cereals. The method is inexpensive and suitable for application at domestic and industrial levels. In addition, it leads to an easily prepared weaning food based on a local and culturally acceptable raw material.

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CAPÍTULO 4 – FERMENTAÇÃO DO SORGO COM BACTÉRIAS LÁCTICAS

1. INTRODUÇÃO

A fermentação é um processo que decorre sob a influência da actividade exercida por enzimas microbianas, sendo os cereais os substratos mais utilizados nos processos fermentativos (Hammes *et al.*, 2005).

A fermentação é a forma mais antiga de biotecnologia alimentar. Um alimento diz-se fermentado quando é submetido à acção de microrganismos com o objectivo de se promover

alterações bioquímicas desejáveis e melhorias significativas na qualidade (Campbell-Platt, 1994).

As fermentações lácticas, juntamente com as alcoólicas, são as mais importantes a nível comercial. Normalmente as fermentações envolvem misturas de microrganismos ou sequências de populações microbianas.

A fermentação tradicional dos alimentos pode resultar no incremento das suas características organolépticas (pelo desenvolvimento de diversos aromas, paladares, cores e texturas), na sua preservação (por acidificação e/ou produção de etanol), no incremento da segurança alimentar (por inibição de microrganismos patogénicos), no enriquecimento biológico (pela produção de proteínas, aminoácidos essenciais, ácidos gordos essenciais e vitaminas), no incremento da biodisponibilidade de alguns componentes (por exemplo pela alteração das propriedades fisico-químicas do amido e por afectar a associação de fibras a vitaminas, minerais e proteínas), na remoção de factores anti-nutricionais (como fitatos, inibidores de enzimas, polifenóis e taninos) e de outros compostos indesejáveis (como micotoxinas e compostos cianogénicos) e na redução dos tempos de cozimento e dos requerimentos energéticos (Chavan e Kadam, 1989b, Hammes *et al.*, 2005, Nout, 1994, Steinkraus, 1995).

A preservação dos alimentos através da fermentação é dependente da oxidação de hidratos de carbono e derivados, da qual normalmente resultam ácidos, álcoois e dióxido de carbono. Estes produtos finais condicionam o crescimento de microrganismos contaminantes. Uma vez que a oxidação é apenas parcial, os alimentos retêm ainda energia suficiente para conferirem benefícios nutricionais ao consumidor (Caplice e Fitzgerald, 1999).

Quando se considera a multiplicidade enorme de alimentos feitos a base de sorgo, é possível concluir que a grande maioria é submetida ao processo de fermentação em pelo menos uma das suas fases de elaboração.

Na fermentação do sorgo, enzimas endógenas, bactérias e leveduras participam individualmente ou em conjugação, contribuindo para a preparação de uma grande variedade de alimentos (Hammes *et al.*, 2005) .

O malte de sorgo é utilizado para iniciar a fermentação espontânea em vários alimentos de sorgo (Mugula *et al.*, 2003c). Para além do aumento da densidade energética dos alimentos, a adição de malte fornece à farinha de sorgo um sistema proteolítico que permite a degradação de proteínas e a sua conversão em pequenos peptídeos e aminoácidos. Deste modo, a adição de malte fornece amino-azoto livre necessário à nutrição dos microrganismos actuantes numa fermentação posterior (Taylor, 1983). A utilização de malte previamente à fermentação fornece assim um sistema enzimático mais diversificado, constituído por enzimas endógenas, de bactérias lácticas e de leveduras (Mugula *et al.*, 2003a, Mugula *et al.*, 2003c). Por outro lado, a adição de malte promove uma descida mais rápida do pH, que inibe o crescimento de microrganismos patogénicos (Mugula *et al.*, 2003c).

A associação de bactérias lácticas e leveduras é comum em vários alimentos e bebidas de sorgo (Bvochora *et al.*, 2005, Mugula *et al.*, 2003a). As bactérias lácticas promovem um ambiente acídico favorável ao crescimento das leveduras. As leveduras, por outro lado, fornecem vitaminas, azoto solúvel e outros factores de crescimento necessários às bactérias, o que sugere a existência de uma associação simbiótica entre bactérias lácticas e leveduras (Bvochora *et al.*, 2005, Mugula *et al.*, 2003b, Mugula *et al.*, 2003c). Este co-metabolismo estável entre bactérias lácticas e leveduras, comum em muitos alimentos, permite a utilização de substratos de outra forma não fermentáveis, aumentando a adaptabilidade dos microrganismos a sistemas alimentares complexos (Mugula *et al.*, 2003a)

Uma vez que a fermentação tradicional do sorgo é muitas vezes realizada a nível doméstico, diferentes processos podem ser utilizados. Consequentemente, verifica-se uma grande heterogeneidade entre os produtos finais e a microflora actuante nem sempre é conhecida, podem estar envolvidos diferentes tipos de microrganismos nestas fermentações.

Na farinha de sorgo utilizada para a preparação do *kisra*, verificou-se a ocorrência de contaminações com bactérias aeróbias. Ao fim de 18 horas de fermentação espontânea do sorgo, estes microrganismos originalmente dominantes desaparecem para dar lugar a uma sucessão de bactérias lácticas tendo-se verificado, num estágio intermédio, a ocorrência de *Enterococcus faecalis*, *Lactococcus lactis* e *Lactobacillus fermentum*. No final da

fermentação, apenas *Lactobacillus fermentum* e *Lactobacillus reuteri* permaneceram como flora dominante. A referida sucessão microbiana parece ser influenciada pelas alterações no pH. À medida que o pH desce, apenas as bactérias tolerantes ao ácido são capazes de sobreviver e crescer (Hamad *et al.*, 1997). No *kisra* também foram encontradas leveduras da espécie *Saccharomyces cerevisiae* (Steinkraus, 1995).

Estudos efectuados no *ogi* revelaram que os organismos predominantes na fermentação são o *L. plantarum*, responsável pela produção de ácido láctico e o *S. cerevisiae*. O *Corynebacterium* é o microrganismo responsável pela hidrólise do amido em ácidos orgânicos, enquanto o *S. cerevisiae* e o *Candida mycoderma* contribuem para o desenvolvimento do aroma e do paladar (Odunfa e Adeyele, 1987).

Outros exemplos de microfloras actuantes encontradas em estudos efectuados em diversos alimentos fermentados de sorgo são:

- *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactococcus lactis*, *Leuconostoc mesenteroides* e *Pediococcus pentosaceus*, isolados em cerveja de sorgo (Pattison *et al.*, 1998);

- Bactérias (*Pediococcus pentosaceus*, *Lactobacillus brevis*, *Lactococcus lactis*, *Lactobacillus cellobiosus*, *Klebsiella osytoca*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter sakasakii*, *Serratia marcescens* e *Serratia odourifera*), fungos (*Penicillium sp.*, *Rhizopus sp.*, *Aspergillus níger*, *Alternaria sp.*, *Fusarium sp.*, e *Mucor sp.*) e leveduras (*Candida parapsilosis*, *Candida orvegensis* e *Rhodotorula glutinis*), isolados nas primeiras 24 horas de fermentação de sorgo para a preparação do *khamir* (Gassem, 1999);

- *Lactobacillus brevis* (mais frequentemente isolado), *Lactobacillus fermentum*, *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lactobacillus plantarum*, *Enterococcus faecium* (segundo mais isolado), *Streptococcus thermophilus*, isolados no bushera (Muyanja *et al.*, 2003);

- *Lactobacillus plantarum* (mais frequentemente isolado), *Lactobacillus brevis*, *Lactobacillus fermentum*, *Lactobacillus cellobiosus*, *Weisella confusa* e *Pediococcus pentosaceus*, *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, *Candida tropicalis* e *Candida pelliculosa*, isolados no *togwa* (Mugula *et al.*, 2003b);

- Corynebacterium, *Lactobacillus plantarum*, *Lactococcus lactis*, *Pediococcus cerevisiae* e *Leuconostoc mesenteroides*, isolados no *kunun zaki* (Gaffa e Gaffa, 2004).

Alguns destes microrganismos, no entanto, são conhecidos pelos seus efeitos toxigénicos. As bactérias dos géneros *Klebsiella*, *Enterobacter* e *Serratia* são patogénicas e responsáveis por vários tipos de infecções (Beck-Sague *et al.*, 1994). Em relação aos fungos, as espécies *Alternaria* e *Aspergillus* produzem micotoxinas e estão associadas a infecções, sinusites, asma e enfisemas pulmonares. As espécies *Penicillium* e *Fusarium* produzem micotoxinas que actuam a nível dos sistemas circulatório, digestivo e nervoso e promovem infecções cutâneas. As espécies *Mucor* e *Rhizopus* possuem propriedades alergénicas, e promovem infecções ao nível dos pulmões, fossas nasais, pele, olhos e cérebro. As leveduras da espécie *Rhodotorula* também são conhecidas pelas suas propriedades alergénicas (Dales *et al.*, 1991).

A grande maioria dos produtos fermentados, incluindo os mais comuns ao mundo ocidental, é dependente de bactérias lácticas para mediação do processo fermentativo. Os produtos finais resultantes do catabolismo dos hidratos de carbono, por parte das bactérias lácticas, contribuem não apenas para a preservação dos alimentos, mas também para o desenvolvimento do sabor, aroma e textura, conferindo assim características únicas aos produtos (Caplice e Fitzgerald, 1999). Por outro lado, a fermentação pode ainda incrementar a qualidade nutricional dos alimentos por melhoria da digestibilidade proteica (aspectos abordados no capítulo anterior). O controlo dos microrganismos ou da sucessão de microrganismos que domina a microflora dos alimentos pode ser assim uma ferramenta bastante útil no desenvolvimento de produtos fermentados de qualidade consistente, microbiologicamente seguros e nutricionalmente enriquecidos. Esta é a base do desenvolvimento de culturas propagadoras.

2. OBJECTIVOS

De forma a evitar variações na qualidade do produto final e problemas associados às contaminações, e por razões inerentes à crescente industrialização e urbanização dos países Africanos, (abordadas no capítulo 2), torna-se importante promover a produção em larga escala de produtos fermentados de sorgo.

Para que se possa promover a transferência do processo fermentativo de uma escala doméstica para uma escala industrial, é necessário proceder à selecção de culturas puras de microrganismos que possam ser utilizadas como culturas propagadoras (*starters*) na fermentação do sorgo.

O principal objectivo deste capítulo foi, partindo de um conjunto alargado de culturas puras de bactérias lácticas, promover a selecção das mais efectivas a serem utilizadas como *starters* para fermentação do sorgo.

Este capítulo teve ainda como objectivo, avaliar as vantagens da utilização conjunta de bactérias lácticas com leveduras e da adição de malte de sorgo previamente à fermentação com bactérias lácticas.

Pretendeu-se ainda estudar os efeitos promovidos pela fermentação do sorgo com a flora microbiana presente em grão de Kefir.

3. ENQUADRAMENTO EXPERIMENTAL

Numa primeira fase deste capítulo, promoveu-se a fermentação do sorgo com diferentes espécies comerciais de bactérias lácticas, simulando o processo tradicional de fermentação. Posteriormente, com recurso a análises químicas e espectroscópicas, avaliou-se os efeitos promovidos por cada uma das espécies isoladas, de forma a seleccionar as mais efectivas com base nos efeitos promovidos em termos de melhoria da qualidade nutricional.

Uma vez seleccionadas as espécies mais efectivas (*Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus*), estudou-se ainda os efeitos promovidos pela

utilização destas espécies conjugadas entre si, ou seja, da utilização de culturas mistas. Promoveu-se assim a fermentação do sorgo com as referidas espécies conjugadas duas a duas e com as três em simultâneo. Neste estudo, incluiu-se ainda uma amostra fermentada de acordo com o processo tradicional (com os microrganismos da flora endógena) para efeitos comparativos.

Tal como foi referido na introdução deste capítulo, a flora microbiana presente em muitos produtos tradicionais fermentados é constituída por bactérias lácticas e leveduras. Quando em conjugação, estes microrganismos por vezes actuam de forma simbiótica conferindo vantagens nutricionais aos alimentos finais. Por outro lado, referiu-se também que o malte de sorgo é muitas vezes adicionado à farinha de forma a iniciar as fermentações espontâneas. Por esta razão, neste capítulo optou-se ainda por, partindo do inóculo final seleccionado, avaliar os efeitos promovidos pela sua utilização conjuntamente com leveduras e pela adição de malte de sorgo previamente à fermentação. Deste modo, foi possível avaliar as vantagens da utilização de um sistema enzimático mais diversificado, constituído por enzimas de bactérias lácticas conjugadas com as das leveduras e com as enzimas endógenas do sorgo. O malte de sorgo utilizado foi o proveniente da germinação do sorgo por cinco dias, uma vez que no capítulo anterior verificou-se ser o inóculo mais adequado.

Com o intuito de prever quais seriam os microrganismos dominantes nestas fermentações mistas, promoveu-se também o acompanhamento do crescimento em meio de cultura das espécies conjugadas entre si, com recurso à espectroscopia de Infra-Vermelho com Transformadas de Fourrier (FT-IR), acoplada à Análise em Componentes Principais (PCA) dos espectros.

Uma vez que os grãos de Kefir apresentam uma flora microbiana complexa, composta por leveduras, *Lactobacillus*, *Lactococcus*, *Streptococcus* e bactérias do ácido acético, optou-se também por utilizar este inóculo na fermentação do sorgo de forma a avaliar as vantagens de um sistema enzimático ainda mais complexo.

3.1. Selecção dos microrganismos

Neste capítulo foram testadas as seguintes espécies microbianas:

- *Lactobacillus plantarum* subsp. *Argentoratensis*,
- *Lactobacillus brevis,*
- *Lactobacillus paracasei subsp. paracasei,*
- *Lactobacillus fermentum,*
- *Pediococcus pentosaceus e*
- *Streptococcus thermophilus*

As referidas espécies foram escolhidas com base na ocorrência com que surgem na literatura como espécies isoladas em produtos fermentados de sorgo. Por outro lado, a escolha assenta ainda no facto de serem bactérias lácticas. Dos diversos estudos publicados em que foi realizada a caracterização da flora actuante em cereais, podem se contar aproximadamente 46 espécies de bactérias do ácido láctico ("lactic acid bacteria" – LAB's), sendo 30 das quais pertencentes ao género *Lactobacillus* (Hammes *et al.*, 2005).

Posteriormente, foram ainda testadas leveduras das espécies *Saccharomyces cerevisiae* e *Issatchenkia orientalis* Kudriavtsev. Ambas as leveduras foram isoladas em todos os estágios da fermentação de vários tipos de *togwa* estudados (Mugula *et al.*, 2003b, Mugula *et al.*, 2003c) e na fermentação do *pito* e do *burukutu* (Jespersen, 2003). Estas espécies têm sido também frequentemente isoladas em fermentações ácidas de diferentes substratos vegetais (Nout, 1980) e são os principais microrganismos isolados no *ogi*, juntamente com o *Lactobacillus plantarum* e *Debaromyces hansenii* (Achi, 2005, Anglani, 1998b).

A espécie *Saccharomyces cerevisiae* é ainda a levedura mais frequentemente isolada em produtos Africanos fermentados (Jespersen, 2003) e aparece ainda como o principal microrganismo isolado no *kisra* (Jespersen, 2003).

4. ESTUDO DOS EFEITOS PROMOVIDOS POR DIFERENTES ESPÉCIES DE BACTÉRIAS LÁCTICAS

"Screening of lactic acid bacteria potentially useful on sorghum fermentation"

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Screening of lactic acid bacteria potentially useful for sorghum fermentation

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Abbreviations Used

ATR - Attenuated Ttotal Reflection

CFU – Cell Forming Units

DNS - 3,5- Dinitrosalicylic Acid

FT-IR – Fourier Transform Infrared

HMW –High Molecular Weight

IVPD – *in vitro* Protein Digestibility

LAB - lactic acid bacteria

OD – Optical Density

PCA – Principal Components Analysis

SDS-PAGE – Sodium Dodecyl Sulfate Polycrylamide Gel Electrophoresis

SNV - Standard Normal Variate

ABSTRACT

A screening of commercial lactic acid bacteria potentially useful in the improvement of sorghum nutritional quality was done. The aim of this study was to test starter cultures to meet the prerequisites for the establishment of small-scale industrial production of sorghum fermented foods in Africa.

Sorghum was fermented with commercial strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Pediococcus pentosaceus* and *Streptococcus thermophilus*.

As a result of sorghum prolamins hydrolysis, an increase in the *in vitro* protein digestibility (IVPD) was promoted. After pepsin digestion, changes occurred on electrophoretic profile of prolamins, with a decrease of almost all fermented samples spots in comparison with unfermented sample. Samples in which the decrease of 45 kDa and 66kDa oligomers was more pronounced, presented higher IVPD.

Fourier Transform Infrared spectroscopy in tandem with multivariate analysis showed starch structural changes on samples fermented with *Lactobacillus brevis*, *Lactobacillus fermentum*, *Streptococcus thermophilus* and *Pediococcus pentosaceus.*

This work demonstrates that all tested bacteria promoted beneficial effects on sorghum nutritional quality and are suitable to be used as commercial starters to industrial applications. *Streptococcus thermophilus*, *Lactobacillus brevis* and *Lactobacillus fermentum* are the most promising starters as they lead to higher IVPD values (46.48, 39.19 and 36.73% respectively).

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench], together with millet, is a major food for around 60 million people concentrated in the inland areas of tropical Africa, who consume them mostly in the form of fermented or unfermented preparations (FAO, 2004). Fermented porridges are prepared in many African countries for human consumption, and appear to be one of the most common types of food prepared from sorghum (Duodu et al., 2003). Heterolactic bacteria are the dominant microorganisms found in sorghum natural fermentation. A wide variety of lactic acid bacteria (LAB) are found in these sorghum products (Calderon et al., 2003).

Fermentation plays an important role on sorghum foods preparation as it provides an improvement of sorghum nutritional quality. Lactic acid fermentation, using natural mixed cultures, has been shown to improve the digestibility of sorghum proteins (Belton and Taylor, 2004). This is of great importance as sorghum is known to become less digestible than other cereals after cooking (Axtell et al., 1981; Eggum, 1983; Hamaker, 1987; MacLean et al., 1981). Fermentation also leads to an increase of sorghum protein content (El Tinay et al., 1979), enhancement of carbohydrates accessibility (Elkhalifa et al., 2004; Elkhalifa et al., 2006), improvement of amino acids balance (Au and Fields, 1981; El Tinay et al., 1979), decrease of anti nutritional factors, like tannins and phytic acid (Osman, 2004), and increase of vitamin content (El Tinay et al., 1979; Kazanas and Fields, 1981).

Household fermentation technologies have been upgraded to an industrial scale in order to provide value added products that meet urban population demand for traditional products (Belton et al., 2004; Gadaga et al., 1999).

One of the prerequisites for the establishment of small-scale industrial production of fermented foods in Africa is the development of starter cultures (Sanni, 1993).

A screening of lactic acid bacteria potentially useful in the improvement of sorghum nutritional quality was done in this work. The aim of this study is to well understand the role played by these LAB's and to evaluate their application for achieving an industrial scale production of sorghum foods.

2. Materials and methods

2.1. Sorghum flour

Sorghum grains, of the Australian variety Jumbo, were purchased in a retail trade and ground with a coffee mill to pass through a 3 x 10^{-4} m sieve.

2.2. Bacterial strains and growth conditions

Lactobacillus plantarum subsp. *argentoratensis* (DSM 16365), *Lactobacillus brevis* (DSM 6235), *Lactobacillus paracasei* subsp. *paracasei* (DSM 20006), *Lactobacillus fermentum* (DSM 20052), *Pediococcus pentosaceus* (DSM 20283) and *Streptococcus thermophilus* (DSM 20617) were obtained from DMSZ (Braunschweig, Germany) in lyophilized form. All these species have been found in traditionally fermented sorghum products (Mugula et al., 2003b; Mugula et al., 2003c; Muyanja et al., 2003).

A MRS broth (Merck - Darmstadt, Germany) was used in their re-hydration. After re-hydration the bacteria were disseminated by streaking on MRS agar (Merck - Darmstadt, Germany) and incubated for 24 h at their optimal growth temperature (37 ºC for *L. fermentum* and *S. thermophilus* and 30 ºC for the other ones).

2.3. Preparation of starter cultures

Aqueous suspensions of starter cultures were prepared from 24 h cultures of each one of the LAB species on agar plates. With sterile loops, LAB cultures was transferred to physiological

serum (NaCl 0.9% (w/v)) and stirred in a vortex. Dilutions were made in order to obtain inoculums containing about 10^7 CFU/mL, determined by optical densities. Optical densities at 600 nm (OD_{600}) were measured using a Shimadzu UV-160A spectrophotometer (Tokyo, Japan). Cellular concentrations of each inoculum were obtained from calibration curves between OD_{600} and the number of colonies/mL determined by standard plate count.

2.4. Flour fermentation

For lactic fermentation, 6 samples of sorghum flour (15 g each) were mixed with sterilized water (1:10 w/v), in sealed E-flasks. These mixtures were boiled, for starch gelatinization, during 1 minute under vigorous stirring prior autoclaving at 121 ºC for 15 min. After cooled, at room temperature (*ca.* 25ºC), each one of these samples was inoculated, respectively, with 5 x 10^{-3} L of *Lactobacillus plantarum* subsp. *argentoratensis, Lactobacillus brevis, Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus fermentum*, *Pediococcus pentosaceus* and *Streptococcus thermophilus* suspensions. All the samples were incubated for five days at the optimal growth temperature of the respective inoculum.

An unfermented control sample was prepared as described before omitting the inoculation step. To keep the same conditions of fermented samples, 5 x 10^{-3} L of physiological serum (NaCl 0.9% w/v) was added.

2.5. Preparation of samples

After fermentations were completed, pH was measured and each of the samples was divided in two portions. The first one was freeze dried and ground again. This portion was kept to determine total protein, soluble proteins, reducing sugars, total sugars and free amino acids. Total protein was determined directly on the freeze dried powder. To analyse each of following soluble components; soluble proteins, reducing sugars, total sugars and free amino

acids; 1 g of the freeze dried samples were mixed with distilled water in a proportion of 1:20 w/v, and magnetic stirred during 1 hour. All samples were centrifuged (2500 x *g* at room temperature) for 3 minutes and the analytes determined in the supernatants. In the case of soluble protein, pH was adjusted to 2 (with HCl) after water addition.

The second portion of each sample was centrifuged at 24000 x *g* during 20 minutes (Sigma 3K30 centrifuge - Osterode am Harz, Germany) and the residues were freeze dried and ground again. These residues were used on the determination of total starch, FT-IR analysis and *in vitro* protein digestibility (IVPD) assay.

2.6. pH measurement

The pH of the samples was measured with a glass electrode.

2.7. Reducing sugars determination

Reducing sugars were determined by the 3,5- dinitrosalicylic acid (DNS) colorimetric method, with glucose as the standard (Miller, 1959). 1 x 10^{-3} L of DNS reagent was added to 1 x 10^{-3} L of sample supernatant. The mix was kept in a boiling water bath for 5 minutes. After cooling to room temperature (*ca.* 25ºC) in a cold water bath, 1 x 10^{-2} L of distilled water was added. The absorbance at 540 nm was measured (Shimadzu UV-160A spectrophotometer), interpolating the value obtained with calculated values for glucose solutions of known concentration. The blanks were prepared by substituting sample solution for distilled water.

2.8. Total sugars determination

A modified phenol-sulphuric acid method was used to determine total sugars present in the samples (Dubois et al., 1956). 1 x 10^{-3} L of 5% phenol was added to 1 x 10^{-4} L of sample. Then, 1 x 10^{-3} L of concentrated sulfuric acid was added and the mixture was kept in a boiling

water bath for 10 minutes. After cooling to room temperature (*ca.* 25ºC), in a cold water bath, the absorbance at 490 nm was measured (Shimadzu UV-160A spectrophotometer - Tokyo, Japan). The amount of sugars was then determined by reference to a standard curve prepared with glucose. The blanks were prepared by substituting sample solution for distilled water.

2.9. Soluble proteins determination

Soluble proteins were determined with a TCA concentration - BCA assay protocol kit for protein determination (Sigma – Missouri, USA).

2.10. Free amino acids determination

The quantitative measurement of free amino acids was made using the ninhydrin reaction (Plummer, 1978). 2 x 10^{-3} L of buffered ninhydrin reagent (0.8 g of ninhydrin and 0.12 g of hydrindantin dissolved in 3 x 10^{-2} L of 2-methoxyethanol plus 1 x 10^{-2} L of acetate buffer 4 M, pH 5.5) were added to 2 x 10^{-3} L of sample and heated in a boiling water bath for 15 minutes. The mixture was cooled to room temperature (*ca.* 25ºC), 3 x 10^{-3} L of 50% ethanol was added and the absorbance was read at 570 nm after 10 minutes (Shimadzu UV-160A spectrophotometer). The amount of amino acids was determined by reference to a standard curve previously prepared with arginine. The blanks were prepared by substituting sample solution for distilled water.

2.11. Total proteins determination

Control and fermented samples were submitted to determination of total N by elementary analysis. The percentage of protein was determined by multiplying for 6.25.

2.12. Total starch Determination

The amount of total starch was determined using a total starch determination kit (Megazyme International Ireland Limited- Wicklow, Ireland).

2.13. Fourier Transform Infrared spectroscopy

Insoluble constituent of control and fermented samples were analyzed by Fourier Transform Infrared (FT-IR) spectroscopy. The FT-IR spectra were obtained using a Golden Gate single reflection diamond attenuated total reflection (ATR) system in a Bruker IFS-55 spectrometer (Ettlingen, Germany). The spectra were recorded in absorbance mode from 4000 to 500 (cm^{-1}), co-adding 128 scans at 8 (cm^{-1}) resolution. Five replicates were collected for each sample. The spectra obtained were transferred into a data analysis package (Barros, 1999). For Principal Component Analysis (PCA) (Jolliffe, 1986), the 1780-800 (cm^{-1}) region was selected as it comprises lipids (1743 (cm^{-1})), protein (1650 – 1500 (cm^{-1})) and starch signals (around 1000 (cm^{-1})), major components of sorghum flour. Each spectrum was SNV corrected (Standard Normal Variate). The PCA allowed the characterization of the sample relationships (scores plans) and the recovery of their sub-spectral profiles (loadings).

2.14. in vitro protein digestibility assay

Control and fermented samples were subjected to *in vitro* protein digestibility assay (IVPD) using pepsin (Sigma - P-7000 - 975 U/(mg) protein) as described by Nunes et al. (2004). However, some modifications on this procedure were done in order to well adapt to fermentation circumstances. This modified procedure takes into account proteins that were digested by microorganisms prior to pepsin digestion. Flour samples (100 mg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 M KH_2PO_4 pH 2 buffer) in a water bath (37^{o}C) for 0 (t0) and 120 minutes (t120). After this period of time, the digestions were stopped by the addition of 1 x 10^{-4} L of 2 M NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, room temperature) for 3 minutes and the supernatants discarded. The residues were washed with 1 x 10^{-3} L of 0.1 M K_2HPO_4 pH 7

buffer, centrifuged and washed again with 1 x 10^{-3} L of water. These residues, with undigested proteins, were freeze-dried and weighed. The content of total N that remains in samples after 0 and 120 minutes of protein digestion was determined by elementary analysis of N and the percentage of protein was determined by multiplying for 6.25.

The percentage of protein digested by pepsin was calculated by difference between protein content after 0 (t0) and 120 (t120) minutes of digestion. The percentage (as a function of total protein) of soluble proteic material prior to pepsin addition was calculated by the sum of total soluble protein and amino acids values. The *in vitro* protein digestibility was defined as the sum of protein solubilized by endogenous and microbial enzymes and the protein digested by pepsin through the follow formula:

$$IVPD = \left(\frac{Soluble\ proteins + Amino\ acids}{Total\ proteins} + \frac{Protein\ content\ (t0) - Protein\ content\ (t120)}{Protein\ content\ (t0)}\right) X\ 100$$

A parallel assay IVPD assay was carried out for sodium dodecyl sulfate polycrylamide gel electrophoresis (SDS-PAGE) analysis.

2.15. SDS-PAGE analysis

To study undigested proteins of the IVPD assay, residues of 0 and 120 minutes of pepsin digestion were submitted to 2 hour protein extraction with 5 x 10^{-4} L 0.0125M $Na_2B_4O_7.10H_2O$ (pH10), 2% (m/v) SDS and 1% (v/v) 2-mercaptoethanol (Hamaker, 1995) to analyze the non-digested proteins. After extraction, the mixtures were centrifuged (2500 x g, room temperature) for 3 minutes. Supernatants, with extracted proteins were prepared for SDS-PAGE by mixing 2 x 10^{-5} L of the protein extract with 1 x 10^{-5} L of SDS-PAGE sample buffer [2% (w/v) SDS, 0.0625 M Tris, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, pH

6.8]. The samples were heated, for 5 minutes, in a boiling water bath and 7 x 10^{-6} L were applied in a 15% acrylamide SDS-PAGE gel (Laemmli method). The gels were run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad - Hercules, USA). Electrophoresis was conducted at 170 V for 1.5 h until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Gels were stained with Coomassie Blue R (GE Healthcare - Uppsala, Sweden) and destained with 40% methanol and 10% acetic acid (Shewry et al., 1995).

2.16. Analysis of SDS-PAGE images

For image analysis each gel image was acquired using the calibrated imaging Gel-Doc (Bio-Rad - Hercules, USA) and analyzed with the Quantity One v4.6 software (Bio-Rad- Hercules, USA). The software allowed background subtraction, automatic band detection and comparative analysis of normalised band optical densities (ODs).

2.17. Statistical analysis

All values are expressed as means and standard deviation for three replicates, with exception of those obtained by SDS-PAGE analysis. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

Changes in pH, free amino acids, soluble and total protein, reducing sugars, total sugars, total starch and *in vitro* protein digestibility are presented in Table 1.

The pH decreased in all fermented samples (Table 1). This is a result of the production of organic acids from sugars. Organic acids detected on previous studies on sorghum fermented flour includes lactic, acetic, formic, succinic, citric, pyruvic, pyroglutamic and uric acids

(Correia et al., 2004; Mugula et al., 2003a; Mugula et al., 2003b; Muyanja et al., 2003). The decrease in pH is observed in all traditional fermented sorghum foods and is important to prevent growth of food poisoning microorganisms (Au et al., 1981).

Fermentation promoted a decrease in reducing and total soluble sugars in almost all samples as a result of their consumption as an energy source. The exception is for sample fermented with *L. brevis* (Table 1). In this sample, the increase in soluble sugars could be related to hydrolysis by bacterial amylases. This assumption is confirmed by the decrease of 34.84% observed in total starch content of sample fermented with *L. brevis* (Table 1). Sugars solubilization promoted by this specie seems to be superior to its consumption needs. Those two different trends in soluble sugars were found on other studies on sorghum fermentation (Correia et al., 2004; El Tinay et al., 1979; Kazanas et al., 1981; Mugula, 1992; Taur et al. 1984).

Table 1 shows that total protein, calculated from the total N determination, increased in all fermented samples. This increase is a result of its concentration, by the consumption of other flour components and its conversion on volatile compounds. The addition of the inoculum also contributes to the increase of protein by the supply of cellular proteins.

Table 1 - Chemical analysis results of the control and fermented samples.

Samples	pH	Free Amino acids (mg/g)[1,2]	Soluble Proteins (mg/g)[1,2]	Total Proteins (%)[1,2]	IVPD (%)[1,2]	Reducing Sugars (mg/g)[1,2]	Total Soluble Sugars (mg/g)1,2	Total Starch (%)[1]
Control Sample	6.41	4.79 ± 0.36	4.35 ± 0.17	9.07 ± 0.15	19.66 ± 0.49	18.81 ± 0.59	96.73 ± 0.55	64.03 ± 2.97
L. plantarum	4.09	3.10 ± 0.16	3.12 ± 0.15	9.46 ± 0.13	32.63 ± 0.56	3.03 ± 0.34	19.54 ± 1.55	65.72 ± 2.07
L. brevis	3.92	7.53 ± 0.14	5.39 ± 0.10	9.85 ± 0.41	39.19 ± 0.33	42.49 ± 0.08	337.81 ± 23.83	41.72 [2] ± 1.77
L. paracasei	3.92	5.37 ± 0.28	1.76 ± 0.07	11.23 ± 0.54	30.76 ± 0.64	4.52 ± 0.11	31.55 ± 1.41	66.34 ± 0.99
L. fermentum	4.61	3.15 ± 0.09	2.06 ± 0.24	9.35 ± 0.35	36.73 ± 0.32	3.26 ± 0.13	17.75 ± 0.62	70.07 ± 2.95
P. pentosaceus	5.09	2.69 ± 0.18	1.29 ± 0.15	9.58 ± 0.28	25.77 ± 0.84	4.86 ± 0.12	28.22 ± 1.98	69.63 ± 2.64
S. thermophilus	4.28	5.88 ± 0.06	1.48 ± 0.11	9.36 ± 0.07	46.48 ± 0.53	13.09 ± 0.20	49.84 ± 3.49	67.95 ± 1.91

[1] Mean of three replicates ± standard deviation.
[2] Differences from control sample were considered significant at $p < 0.05$.

Soluble proteins and free amino acids of sample fermented with *L. brevis* increased 23.91% and 57.20% respectively. This increase is due to hydrolysis of insoluble proteins by bacterial proteases. The microorganisms are able to hydrolyze proteins into usable amino acids and peptides. Additionally, during their growth cycle, they can synthesize amino acids from metabolic intermediates (Au et al., 1981). It seems that *L. brevis* presents amylolytic and proteolytic activities simultaneously. In agreement with some authors who suggested that starch granules are completely enclosed in a very compact protein matrix, which can restrict starch granules from fully gelatinization, the pronounced proteolytic attack to the proteins could make starch more accessible to bacterial amylases, and should be an explanation, as well, for the decrease of starch level in sample fermented with *L. brevis*. (Chandrashekar and Kirleis, 1988; Elkhalifa et al., 2006; Elkhalifa et al., 2004).

Samples fermented with *L. plantarum*, *L. paracasei*, *L. fermentum*, *P. pentosaceus* and *S. thermophilus* present a different pattern of soluble proteins. As we can see in Table 1, there is a decrease in soluble proteins with these inoculums. This result indicates that soluble proteins were the principal target of proteolysis for these bacteria. Soluble proteins of these samples were hydrolyzed in amino acids by bacterial proteases and peptidases. These amino acids can be readily utilized by the microflora during their metabolic activity. As was said before, microorganisms can also promote a *de novo* production of amino acids. When the amino acids increment (promoted by the referred production and/or by proteins hydrolysis) is superior to the amino acids utilization, it is observed a final increase in free amino acid levels. This is the case of samples fermented with *L. brevis*, *L. paracasei* and *S. thermophilus*, where an increase in free amino acid levels was observed. When samples were fermented with *L. plantarum*, *L.*

fermentum and *P. pentosaceus* a decrease in free amino acid levels occurred indicating amino acids consumption by these species.

In vitro protein digestibility assays using enzymes (such as pepsin) do not take into account proteins previously digested by microorganisms. These currently used methods are not applicable to fermentation process as they only give us information about pepsin accessibility to the remained proteins (proteins not digested by microorganisms). The previously described method used in this work is suitable for the determination of protein digestibility of fermented sorghum as it comprises protein digested by pepsin and microorganisms. The obtained IVPD values increased in all fermented samples (Table 1). The increase in proteins digestibility has been reported for some sorghum traditional fermented foods (Axtell et al., 1981; Chavan et al., 1988; Graham et al., 1986; Hassan and El Tinay, 1995; Moneim et al., 1995; Osman, 2004). The highest IVPD values were observed on samples fermented with *S. thermophilus*, *L. brevis* and *L. fermentum*. With the referred inoculums, proteins digestibility increases from 19.66% to 46.48, 39.19 and 36.73% respectively.

Insoluble protein that remains on the residue after 0 (t0) and 120 minutes (t120) of pepsin digestion were extracted and analyzed by SDS-PAGE. As reported previously by Nunes et al. (2004), a typical electrophoregram of prolamins extracted from sorghum flour presents spots that correspond to high molecular weight (HMW) aggregates, 66 kDa trimer, 45 kDa dimer and γ, α, β monomers (Figure 1).

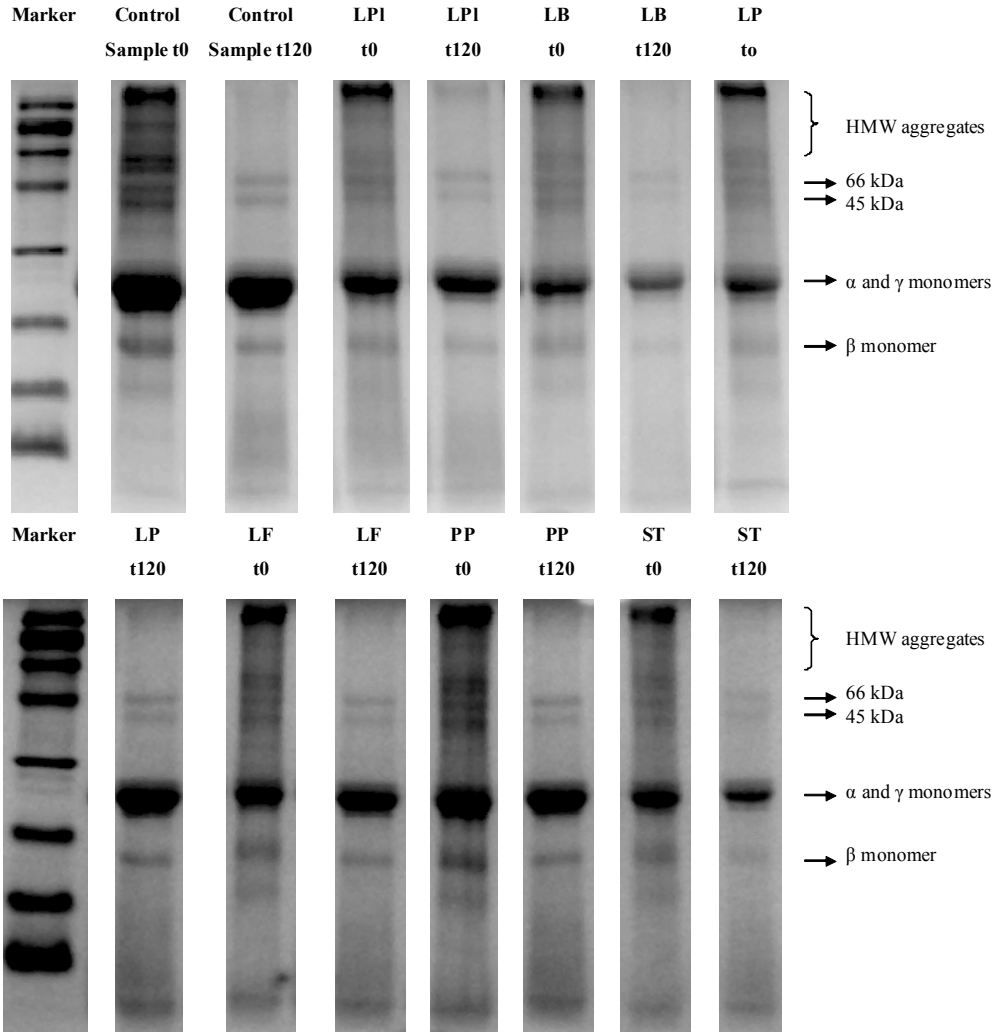


Figure 1 - Electrophoretic gels correspondent to 0 (t0) and 120 (t120) minutes of pepsin digestion of control sample and samples fermented with *L. plantarum* (LPl), *L. brevis* (LB), *L. paracasei* (LP), *L. fermentum* (LF), *P. pentosaceus* (PP) and *S. thermophilus* (ST).

The optical densities of each one of these electrophoretic spots of fermented samples were determined and expressed as a fraction of the highest spot of the control sample (α and γ spot of control sample at 0 minutes of pepsin digestion) (Table 2).

Table 2 – Optical densities of electrophoretic spots of α and γ monomers, β monomers, 45 and 66 kDa oligómeros and HMW, extracted after pepsin digestion of control and fermented samples at 0 (t0) and 120 minutes (t120).

Samples	α and γ monomers [1]	β monomer [1]	45 kDa oligomer [1]	66 kDa oligomer [1]	HMW aggregates [1]
Control Sample t0	1.00	0,25	0.27	0.27	1.06
Control Sample t120	0.97	0.21	0.19	0.19	n.d. [2]
***L. brevis* t0**	0.40	0.19	0.21	0.22	0.91
***L. brevis* t120**	0.35	0.13	0.14	0.15	n.d. [2]
***L. plantarum* t0**	0. 43	0.21	0.22	0.23	0.94
***L. plantarum* t120**	0.42	0.17	0.16	0.17	n.d. [2]
***L. paracasei* t0**	0.38	0.18	0.20	0.21	0.89
***L. paracasei* t120**	0.55	0.20	0.16	0.16	n.d. [2]
***L. fermentum* t0**	0.50	0.20	0.21	0.21	0.95
***L. fermentum* t120**	0.52	0.18	0.15	0.15	n.d. [2]
***P. pentosaceus* t0**	0.54	0.23	0.23	0.23	1.02
***P. pentosaceus* t120**	0.55	0.19	0.15	0.16	n.d. [2]
***S. thermophilus* t0**	0.47	0.19	0.19	0.19	0.86
***S. thermophilus* t120**	0.41	0.15	0.13	0.13	n.d. [2]

[1] Optical densities of spots expressed as a fraction of the higher optical density (spot of α and γ monomers of control sample t0)
[2] n.d. = not detectable. Optical densities values below detection limits.

The observation of protein profiles at 0 minutes of pepsin digestion (t0) gives us the information about the effects of fermentation on sorghum proteins (Figure 1). As we can see in Table 2, there was a decrease in spots correspondent to HMW aggregates, α and γ, β, 45 kDa and 66 kDa prolamins for all fermented samples in comparison to control sample. This indicates that these insoluble proteins were hydrolyzed by bacterial proteases. We can conclude that, although soluble proteins seems to be the principal target of proteolysis for some species, all the tested species are able to hydrolyze insoluble proteins.

After 120 minutes of pepsin digestion, a decrease in all electrophoretic spots was observed, with exception of the corresponding to α and γ monomers of the sample fermented with *L. paracasei*, *L. fermentum* and *P. pentosaceus*, and of the corresponding to β monomer of the sample fermented with *L. paracasei*, for which an increase was observed (Table 2). The S-S bonds of HMW aggregates were cleaved, the oligomers were disrupted and their constituents were revealed leading to an increase of α, γ or β monomers in these samples. Similar results were found by Nunes et al. (2005).

The higher decrease observed after 120 minutes of pepsin digestion was on HMW aggregates where optical densities drop to values below the detectable limit (Table 2).

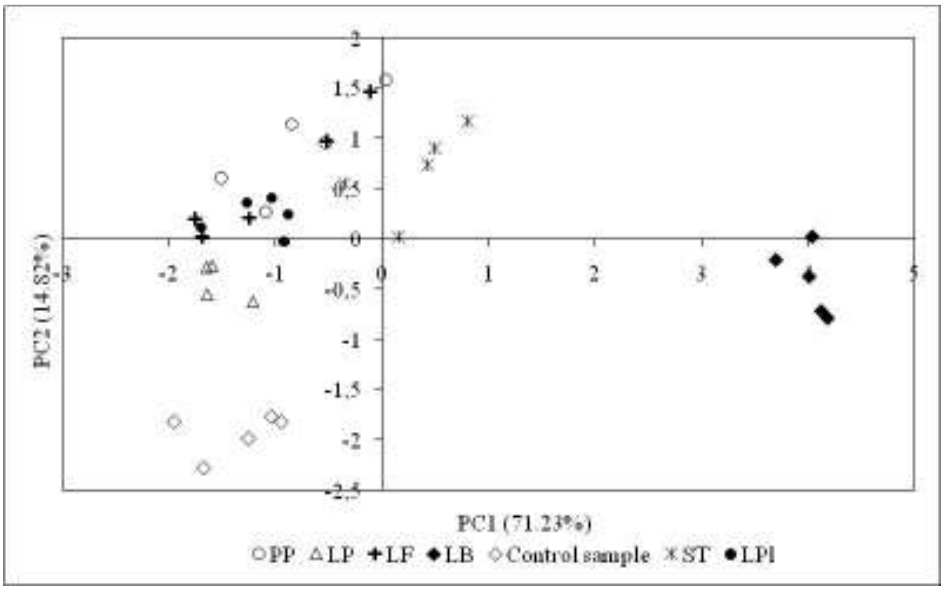
After 120 minutes of digestion, spots corresponding to 45 kDa and 66 kDa prolamins presented the lowest optical densities on samples fermented with *S. thermophilus* (0.13 and 0.13, respectively) *L. brevis* (0.14 and 0.15) and *L. fermentum* (0.15 and 0.15) (Table 2). It is interesting that the samples which presented a decrease of the 45 kDa and 66 kDa oligomers have higher IVPD values (Table 1).

The appearance of a 45 kDa prolamins with cooking was previously reported in other studies (Duodu et al., 2002; Nunes et al., 2004). Nunes et al. (2004) found that part of this

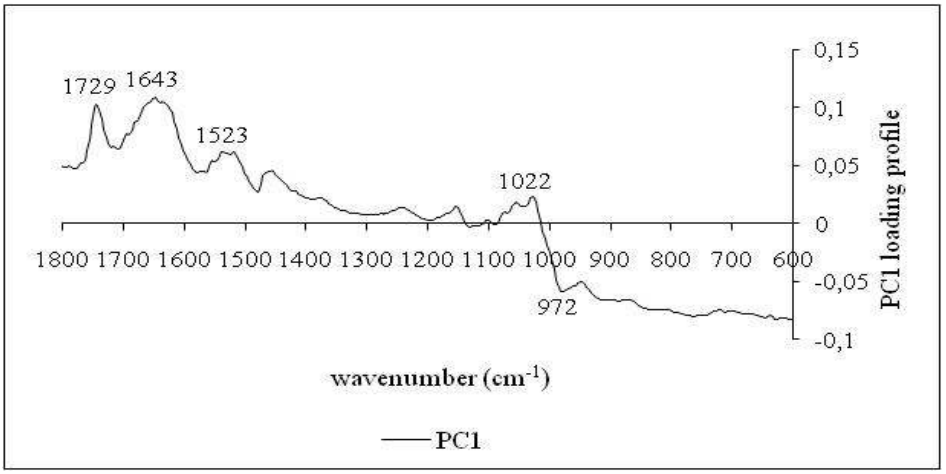
protein resists both 120 minutes pepsin digestion and reducing conditions used in the proteins extraction. According to these authors, formation of un-reducible 45 kDa proteins is more evident in sorghum than in maize and seems to be associated with the decrease in sorghum digestibility with cooking. These findings lead us to conclude that fermentation promotes changes that increase prolamins digestibility which is related with the decrease in 45 kDa oligomers.

The principal components analysis (PCA) of FT-IR spectra shows in which degree fermentation with different inoculums affects sorghum macromolecules. The scores scatter plot of samples (Figure 2a) shows that, along PC1 axis, sample fermented with *L. brevis* (PC1 (+)) is separated from control sample and samples fermented with *L. plantarum*, *L. paracasei*, *L. fermentum* and *P. pentosaceus* (PC1(-)). PC1 loading profile showed that the sample fermented with *L. brevis* is characterized by absorption bands corresponding to proteins (1643 and 1523 (cm^{-1})) and lipids (1729 (cm^{-1})) (Figure 2b), indicating a superior content of these macromolecules in this sample. This could be a result of protein and lipids concentration due to dry matter loss promoted by the observed decrease of starch content (Table 1). When starch is enzymaticaly attacked by α-amilases, the maximum of its characteristic peak shifted from 976 to 1018 (cm^{-1}) (Correia et al., 2004). In the PC1 loading profile one can also see a shift on the maximum of the starch peak from 972 to 1022 (cm^{-1}) sustaining the findings of starch hydrolysis by *L. brevis*. Sample fermented with *S. thermophilus* is also situated on PC1 (+), however, the proximity to PC1 axis origin indicates that this sample has a lower influence of PC1 loading profile.

a)


PC2 (14.82%)
PC1 (71.23%)
PP LP LF LB Control sample ST LPl

b)


1729
1643
1523
1022
972
PC1 loading profile
wavenumber (cm-1)
PC1

c)

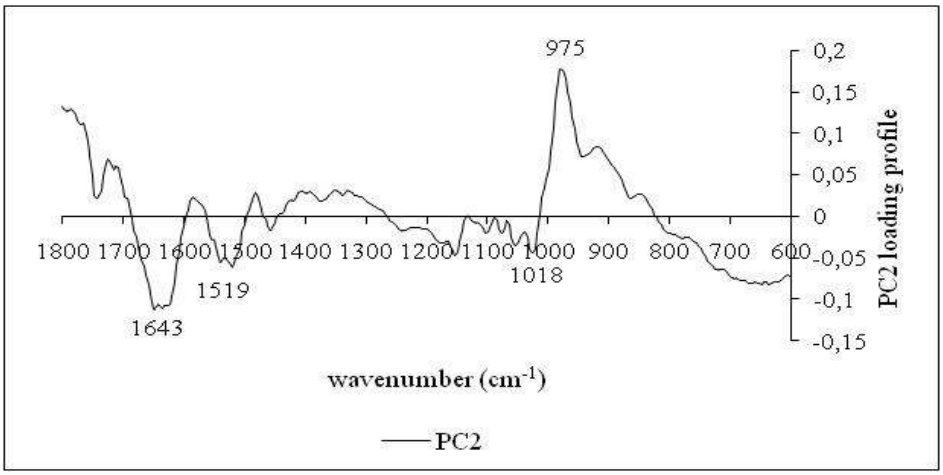


Figure 2 – (a) Scores scatter plot (PC1 *vs*. PC2), **(b)** PC1 Loadings profile and **(c)** PC2 Loadings profile, of the FT-IR spectra of control sample and samples fermented with *L. plantarum* (LPl), *L. brevis* (LB), *L. paracasei* (LP), *L. fermentum* (LF), *P. pentosaceus* (PP) and *S. thermophilus* (ST).

PCA also separated control sample, located on PC2 (-), from samples fermented with *L. fermentum*, *P. pentosaceus* and *S. thermophilus*, situated on PC2 (+) (Figure 2a). As we can see in PC2 loading profile, control sample is characterized by proteins absorption bands at 1643 and 1519 (cm^{-1}) (Figure 2c), which reflects the superior insoluble protein content of this sample. This is a result of protein hydrolysis in samples fermented with *L. fermentum*, *P. pentosaceus* and *S. thermophilus*.

Once again, the maximum of starch peak shifted from 975 to 1018 (cm^{-1}) as a result of starch hydrolysis promoted by fermentation (Figure 2c). This means that although starch content of samples fermented with *L. fermentum*, *P. pentosaceus* and *S. thermophilus* did not significantly decreased, starch was hydrolyzed by these species. The decrease in starch content is probably been masked by consumption of non-starch components leading to starch

concentration in samples. However, information given by the PC1 (Figure 2a and b) show that starch attack promoted by *L. brevis* occur in a higher extension.

The proximity of samples fermented with *L. brevis*, *L. plantarum* and *L. paracasei*, indicates that these sample have lower influence of PC2 loading profile (Figure 2a).

4. Conclusions

All the tested strains of commercial lactic acid bacteria are able to fermented sorghum flour. Fermentations with the tested strains of *Lactobacillus plantarum*, *Lactobacillus brevis*, *Lactobacillus paracasei*, *Lactobacillus fermentum*, *Pediococcus pentosaceus* and *Streptococcus thermophilus* leaded to the organic acids production and the consequent decrease in pH, sugars metabolism, protein hydrolysis and increase in IVPD values, characteristics of sorghum traditional fermented foods.

The most effective species were *Streptococcus thermophilus*, *Lactobacillus brevis* and *Lactobacillus fermentum*, which presented stronger amylolytic and proteolytic activities resulting on a higher increase in IVPD values. It was also found that decrease in 45 and/or 66 kDa oligomers may be related to the improvement of sorghum prolamins digestibility.

This works provides an approach to the possibility to use commercial strains of acid lactic bacteria to the industrial scale production of sorghum fermented foods with improved nutritional qualities. However, further work must be done to evaluate advantages to use these species in co-cultures and to check organoleptic characteristics of the final product, consumers' acceptation and other important nutritional requisites, like amino acids and bulk density.

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5. FERMENTAÇÃO DO SORGO COM CULTURAS MISTAS DE BACTÉRIAS LÁCTICAS

"Sorghum fermentation with different combinations of lactic acid bacteria"

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Sorghum fermentation with different combinations of lactic acid bacteria

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Abbreviations Used

ATR - Attenuated Total Reflection

CFU – Cell Forming Units

DNS - 3,5- Dinitrosalicylic Acid

FT-IR – Fourier Transform Infrared

HMW –High Molecular Weight

IVPD – *in vitro* Protein Digestibility

LAB - lactic acid bacteria

LB + LF - *Lactobacillus brevis* and *Lactobacillus fermentum*

LB + ST - *Lactobacillus brevis* and *Streptococcus thermophilus*

LB + LF + ST - *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*

LF + ST - *Lactobacillus fermentum* and *Streptococcus thermophilus*

OD – Optical Density

SDS-PAGE – Sodium Dodecyl Sulfate Polycrylamide Gel Electrophoresis

ABSTRACT

Single and mixed cultures of lactic acid bacteria (*Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*) were used to assess their effects on sorghum nutritional quality. The ultimate aim of this study is to propose a starter culture for industrial production of sorghum fermented foods in Africa. In addition, traditional fermentation was also simulated to establish comparison.

Fourier Transform Infrared spectroscopy showed that all tested inoculums promoted starch structural changes and stronger proteolysis on samples fermented with *L. fermentum* and *S. thermophilus*

As a consequence of protein and/or starch hydrolysis, an increase in *in vitro* protein digestibility (IVPD) values was noticed in all fermented samples. Fermentation with an inoculum composed of *Streptococcus thermophilus*, *Lactobacillus brevis* and *Lactobacillus fermentum* has giving an IVPD value of 65%, higher than the obtained from traditional fermentation (52%). Sodium dodecyl sulfate polycrylamide gel electrophoresis showed that the higher IVPD value obtained with this mixed inoculum may be related to the decrease in 45 kDa oligomers.

This work showed that conjugation of the three studied species has the potential of being used as starter in industrial applications. The amylolitic activity of *L. brevis* conjugated with the proteolytic activity of *L. fermentum* and *S. thermophilus* promoted beneficial effects on sorghum quality.

1. Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a common substrate for the production of a wide variety of fermented products in Africa. Besides cultural reasons, fermentation plays an important role on sorghum utilization as it improves sorghum nutritional value and enhances its sensorial properties (Murty and Kumar, 1995).

However, since the preparation of sorghum fermented products is still a traditional family art and the fermentation process is done by uncontrolled inoculation, some variations occur in the quality and stability of the sorghum fermented products (Sanni, 1993).

In developing countries, the demand of this cereal is increasing due to the population growing and to their policy to enhance its processing and industrial utilization (Akintayo and Sedgo, 2001; Dicko et al., 2006).

The migration of the rural population to urban areas is associated with changes in lifestyle and food quality demanding. There is, hence, a need to upgrade household fermentation technologies to an industrial scale in order to provide sorghum products of consistent quality, safety and stability.

According to Sanni (1993), the development of starter cultures is one of the prerequisites for the establishment of small-scale industrial production of fermented foods in Africa.

In this study, single and mixed cultures of lactic acid bacteria (*Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*) were tested as starter cultures for a sorghum flour controlled fermentation. The purpose of this work was to evaluate the effects promoted by these lactic bacteria on sorghum nutritional quality and to propose a starter culture for industrial applications.

2. Experimental

2.1. Sorghum flour

Sorghum grains, of the Australian variety Jumbo, were purchased in a retail trade and ground with a coffee mill to pass through a 3 x 10^{-4} m sieve.

For the traditional fermentation a South African commercial flour (with sorghum endogenous microorganisms) was used (King Korn Mabele – King Food Corporation, South Africa).

2.2. Bacterial strains and growth conditions

Lactobacillus brevis (DSM 6235), *Lactobacillus fermentum* (DSM 20052) and *Streptococcus thermophilus* (DSM 20617) were obtained from DMSZ (Braunschweig, Germany) in a lyophilized form. All these species have been found in sorghum fermented products (Mugula et al., 2003b; Mugula et al., 2003c; Muyanja et al., 2003).

After re-hydration in MRS broth (Merck - Darmstadt, Germany), the bacteria were seeded on MRS agar (Merck - Darmstadt, Germany) by spreading and incubated for 24 h at their optimal growth temperature (37 ºC for *L. fermentum* and *S. thermophilus* and 30 ºC for *Lactobacillus brevis*).

2.3. Preparation of starter cultures

Aqueous suspensions of starter cultures were prepared from 24 h cultures of each one of the bacteria species on agar plates. With sterile loops, bacteria cultures were transferred to physiological serum (NaCl 0.9% w/v) and stirred in a vortex. Dilutions were made in order to obtain inoculums containing about 10^7 CFU/mL, determined by optical densities measurement. Optical densities at 600 nm (OD_{600}) were measured using a Shimadzu UV-160A spectrophotometer (Tokyo, Japan). Cellular concentrations of each inoculum were

obtained from calibration curves between OD_{600} and the number of colonies/mL determined by standard plate count.

2.4. Flour fermentation

For controlled fermentation with single cultures, 3 samples of sorghum flour (15 x 10^{-3} kg each) were mixed with sterilized water (1:10 w/v), in sealed E-flasks. These mixtures were boiled for 1 minute under vigorous stirring in order to homogenize the gelatinization process. These samples were then autoclaved at 121 ºC for 15 min. After cooled, at room temperature (*ca.* 25ºC), each one of these samples was inoculated, respectively, with 5 x 10^{-3} L of *Lactobacillus brevis* (LB), *Lactobacillus fermentum* (LF) and *Streptococcus thermophilus* (ST). The samples were then incubated for five days at the optimal growth temperature of the respective inoculum.

For controlled fermentation with mixed commercial cultures, 4 samples of sorghum flour (15 x 10^{-3} kg each) were mixed with sterilized water (1:10 w/v), in sealed E-flasks. These mixtures were boiled and autoclavated as described before. Different combinations of commercial bacterial species were inoculated in each sample: *Lactobacillus brevis* + *Lactobacillus fermentum* (LB+LF); *Lactobacillus brevis* + *Streptococcus thermophilus* (LB+ST); *Lactobacillus fermentum* + *Streptococcus thermophilus* (LF+ST) (inoculated with 2.5 x 10^{-3}L of each bacterial suspension) and *Lactobacillus brevis* + *Lactobacillus fermentum* + *Streptococcus thermophilus* (LB+LF+ST) (inoculated with 1.667 x 10^{-3} L of each bacterial suspension). All the samples were incubated for five days at 30ºC (optimal growth temperature of *Lactobacillus brevis*) with exception of the sample fermented with *Lactobacillus fermentum* + *Streptococcus thermophilus*, which was incubated at 37ºC (optimal growth temperature for both species).

For the simulation of traditional fermentation, Mabele sorghum flour (5 x 10^{-3} kg) was mixed with 5 x 10^{-2} L of cool sterilized water. This mixture was left to ferment, with the natural microflora, at room temperature (*ca.* 25ºC) until pH ≈ 4, which occurred after 1 day. Sorghum flour (15 x 10^{-3} kg of Jumbo variety) was mixed with sterilized water (1:10 w/v), in sealed E-flasks. Previously fermented flour, with endogenous microorganisms, was added to act as a starter for this fermentation. This mixture was left to ferment at room temperature (*ca.* 25ºC). After one day of fermentation (time to reach pH≈4), 5 x 10^{-3} L of physiological serum (NaCl 0.9% w/v) was added and the sample was boiled and autoclavated, as described before, to bring starch to the same conditions of controlled fermentations.

An unfermented sample (control sample) was prepared as described for controlled fermentations, except for the inoculation step. To keep the same conditions of fermented samples, 5 x 10^{-3} L of physiological serum (NaCl 0.9% w/v) was added.

2.5. Samples preparation

After fermentations were completed, pH was measured and each of the samples was divided in two portions. The first one was freeze dried and ground again. This portion was kept to determine total protein, soluble proteins, reducing sugars, total sugars and free amino acids. Total protein was determined directly on the freeze dried powder. To analyse each of following soluble components; soluble proteins, reducing sugars, total sugars and amino acids; 1 x 10^{-3} kg of the freeze dried samples were mixed with distillated water in a proportion of 1:20 w/v, and magnetic stirred during 1 hour. All samples were centrifuged (2500 x *g* at room temperature) for 3 minutes and the analytes determined in the supernatants. In the case of soluble protein, pH was adjusted to 2 (with HCl) after water addition.

The second portion of each sample was centrifuged at 24000 x *g* during 20 minutes (Sigma 3K30 centrifuge - Osterode am Harz, Germany) and the residues were freeze dried and ground again. These residues were used for the determination of total starch, FT-IR analysis, *in vitro* protein digestibility (IVPD) assay and SDS-PAGE analysis of insoluble proteins.

2.6. Reducing sugars determination

Reducing sugars were determined by the 3,5- dinitrosalicylic acid (DNS) colorimetric method, with glucose as the standard (Miller, 1959). 1 x 10^{-3} L of DNS reagent was added to 1 x 10^{-3} L of sample supernatant. The mix was kept in a boiling water bath for 5 minutes. After cooling to room temperature (*ca.* 25ºC) in a cold water bath, 1 x 10^{-2} L of distillated water was added. The absorbance at 540 nm was measured (Shimadzu UV-160A spectrophotometer), interpolating the value obtained with calculated values for glucose solutions of known concentration. The blanks were prepared by substituting sample solution by distilled water.

2.7. Total sugars determination

A modified phenol-sulphuric acid method was used to determine total sugars present in the samples (Dubois et al., 1956). 1 x 10^{-3} L of 5% phenol was added to 1 x 10^{-4} L of sample. Then, 1 x 10^{-3} L of concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 10 minutes. After cooling to room temperature (*ca.* 25ºC), in a cold water bath, the absorbance at 490 nm was measured (Shimadzu UV-160A spectrophotometer - Tokyo, Japan). The amount of sugars was then determined by reference to a standard curve prepared with glucose. The blanks were prepared by substituting sample solution by distilled water.

2.8. Soluble proteins determination

Soluble proteins were determined with a TCA concentration - BCA assay protocol kit for protein determination (Sigma – Missouri, USA).

2.9. Free amino acids determination

The quantitative measurement of free amino acids was made using the ninhydrin reaction (Plummer, 1978). Two mL of buffered ninhydrin reagent (8×10^{-4} kg of ninhydrin and 12×10^{-5} kg of hydrindantin dissolved in 3×10^{-2} L of 2-methoxyethanol plus 1×10^{-3} L of acetate buffer 4 M, pH 5.5) were added to 2×10^{-3} L of sample and heated in a boiling water bath for 15 minutes. The mixture was cooled to room temperature (*ca.* 25ºC), 3×10^{-3} L of 50% ethanol was added and after 10 minutes the absorbance was read at 570 nm (Shimadzu UV-160A spectrophotometer). The amount of amino acids was determined by reference to a standard curve previously prepared with arginine. The blanks were prepared by substituting sample solution by distilled water.

2.10. Total proteins determination

Control and fermented samples were submitted to determination of total N by elementary analysis and percentage of protein was determined by multiplying with 6.25.

2.11. Total starch Determination

The amount of total starch was determined using a total starch determination kit (Megazyme International Ireland Limited- Wicklow, Ireland).

2.12. Fourier Transform Infrared spectroscopy

Insoluble constituent of control and fermented samples were analyzed by Fourier Transform Infrared (FT-IR) spectroscopy. The FT-IR spectra were obtained using a Golden Gate single reflection diamond attenuated total reflection (ATR) system in a Bruker IFS-55 spectrometer

(Ettlingen, Germany). The spectra were recorded in absorbance mode from 4000 to 500 (cm^{-1}), co-adding 128 scans at 8 (cm^{-1}) resolution. Five replicates were collected for each sample. Samples spectra were normalized and presented as means of the five replicates

2.13. in vitro protein digestibility assay

Control and fermented samples were subjected to *in vitro* protein digestibility assay (IVPD) using pepsin (Sigma - P-7000 - 975 U/mg protein) as described by Nunes et al. (2004a) but some modifications were done in order to be adapted to fermentation processing. This modified procedure takes into account proteins that were digested by microorganisms prior to pepsin digestion. Flour samples (1×10^{-1} kg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 M KH_2PO_4 pH 2 buffer) in a water bath (37^oC) for 0 (t0) and 120 minutes (t120). After this period of time, the digestions were stopped by the addition of 1×10^{-4} L of 2 M NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, room temperature) for 3 minutes and the supernatants discarded. The residues were washed with 1×10^{-3} L of 0.1 M K_2HPO_4 pH 7 buffer, centrifuged and washed again with 1×10^{-3} L of water. These residues, with insoluble undigested proteins, were freeze-dried and weighed. The content of total N that remains in control samples (t0) and in samples after 120 minutes (t120) of protein digestion was determined by elementary analysis of N.

The proportion (as a function of initial total protein) of soluble proteic material prior to pepsin addition was calculated by the sum of soluble protein and amino acids values.

The percentage of insoluble proteins digested by pepsin was calculated by the difference between insoluble protein content at 0 (t0) and 120 (t120) minutes of digestion. The *in vitro* protein digestibility was defined as the sum of protein solubilized by endogenous and

microbial enzymes and the proportion of insoluble protein digested by pepsin through the follow formula:

$$IVPD = \left(\frac{SP + AA}{TP} + \frac{\text{Protein content (t0)} - \text{Protein content (t120)}}{\text{Protein content (t0)}} \right) x\,100$$

Where SP is the soluble proteins content prior pepsin digestion; AA is the free aminoacids content prior pepsin digestion; TP is the total protein; Protein content (t0) – Protein content (t120) is the content of proteins solubilized by pepsin in the IVPD assay and Protein content (t0) in the content of insoluble proteins prior IVPD assay

2.15. SDS-PAGE analysis

To study insoluble proteins, residues were submitted to 2 hour protein extraction with 5 x 10^{-4} L 0.0125M $Na_2B_4O_7.10H_2O$ (pH10), 2% (m/v) SDS and 1% (v/v) 2-mercaptoethanol (Hamaker, 1995). After extraction, the mixtures were centrifuged (2500 x g, room temperature) for 3 minutes. Supernatants, with extracted proteins were prepared for SDS-PAGE by mixing 2 x 10^{-5} L of the protein extract with 1 x 10^{-5} L of SDS-PAGE sample buffer [2% (w/v) SDS, 0.0625 M Tris, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, pH 6.8]. The samples were heated, for 5 minutes; in boiling water bath and 7 x 10^{-6} L were applied in a 15% acrylamide SDS-PAGE gel (Laemmli method). The gels were run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad - Hercules, USA). Electrophoresis was conducted at 170 V for 1.5 h until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Molecular weight markers were used to compare electrophoretic mobility of proteins (Broad Range Markers – BioRad). Gels were stained with Coomassie Blue R (GE Healthcare - Uppsala, Sweden) and destained with 40% methanol and 10% acetic acid (Shewry et al., 1995).

2.16. Analysis of SDS-PAGE images

For image analysis each gel image was acquired using the calibrated imaging Gel-Doc (Bio-Rad - Hercules, USA) and analyzed with the Quantity One v4.6 software (Bio-Rad- Hercules, USA). The software allowed background subtraction, automatic band detection and comparative analysis of normalised band optical densities (ODs).

2.17. Statistical analysis

All values are expressed as means and standard deviation for three replicates, with exception of those obtained by SDS-PAGE analysis. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

Changes in pH, free amino acids, soluble and total protein, reducing sugars, total soluble sugars, total starch and *in vitro* protein digestibility are presented in Table 1.

The pH decreased in all fermented samples as a result of production of organic acids (Table 1). Organic acids produced from sorghum flour fermentation were found to be predominantly lactic, acetic, formic, succinic, citric, pyruvic, pyroglutamic and uric acids (Correia et al., 2004; Mugula et al., 2003a; Mugula et al., 2003b; Muyanja et al., 2003). The decrease in pH observed in the traditional fermented sample is important to prevent pathogenic bacteria growth in the obtained fermented product (Au and Fields, 1981).

Table 1 - Chemical analysis results of the control and fermented samples.

Samples	pH	Free Amino acids (mg/g)[1,2]	Soluble Proteins (mg/g)[1,2]	Total Proteins (%)[1,2]	IVPD (%)[1,2]	Reducing Sugars (mg/g)[1,2]	Total Soluble Sugars (mg/g)[1,2]	Total Starch (%)[1]
Control Sample	6.41	4.79 ± 0.36	4.35 ± 0.17	9.07 ± 0.15	19.66 ± 0.49	18.81 ± 0.59	96.73 ± 0.55	64.03 ± 2.97
L. brevis	3.92	7.53 ± 0.14	5.39 ± 0.10	9.85 ± 0.41	39.19 ± 0.33	42.49 ± 0.08	337.81 ± 23.83	41.72 [2] ± 1.77
L. fermentum	4.61	3.15 ± 0.09	2.06 ± 0.24	9.35 ± 0.08	36.73 ± 0.32	3.26 ± 0.13	17.75 ± 0.62	70.07 ± 2.95
S. thermophilus	4.28	5.88 ± 0.06	1.48 ± 0.11	9.36 ± 0.07	46.48 ± 0.53	13.09 ± 0.20	49.84 ± 3.49	67.95 ± 1.91
LB + LF	3.58	8.05 ± 0.14	1.92 ± 0.50	14.59 ± 0.44	31.38 ± 0.72	30.33 ± 0.19	171.13 ± 2.71	50.88 [2] ± 1.94
LB + ST	3.61	3.59 ± 0.25	2.07 ± 0.32	13.08 ± 0.03	41.31 ± 0.57	14.73 ± 1.15	145.72 ± 6.79	63.52 ± 1.35
LF + ST	3.80	3.53 ± 0.12	2.53 ± 0.02	12.63 ± 0.30	34.56 ± 0.57	17.25 ± 0.54	72.44 ± 2.72	68.87 ± 1.57
LB + LF + ST	4.29	4.05 ± 0.16	1.21 ± 0.08	13.30 ± 0.78	64.75 ± 0.51	9.93 ± 0.46	124.14 ± 3.25	65.88 ± 0.88
Endogenous Microflora	3.33	5.77 ± 0.03	1.15 ± 0.43	13.06 ± 0.07	52.43 ± 0.62	8.35 ± 0.07	51.66 ± 5.80	69.09 ± 2.50

[1] Mean of three replicates ± standard deviation
[2] Differences from control sample were considered significant at $p < 0.05$.

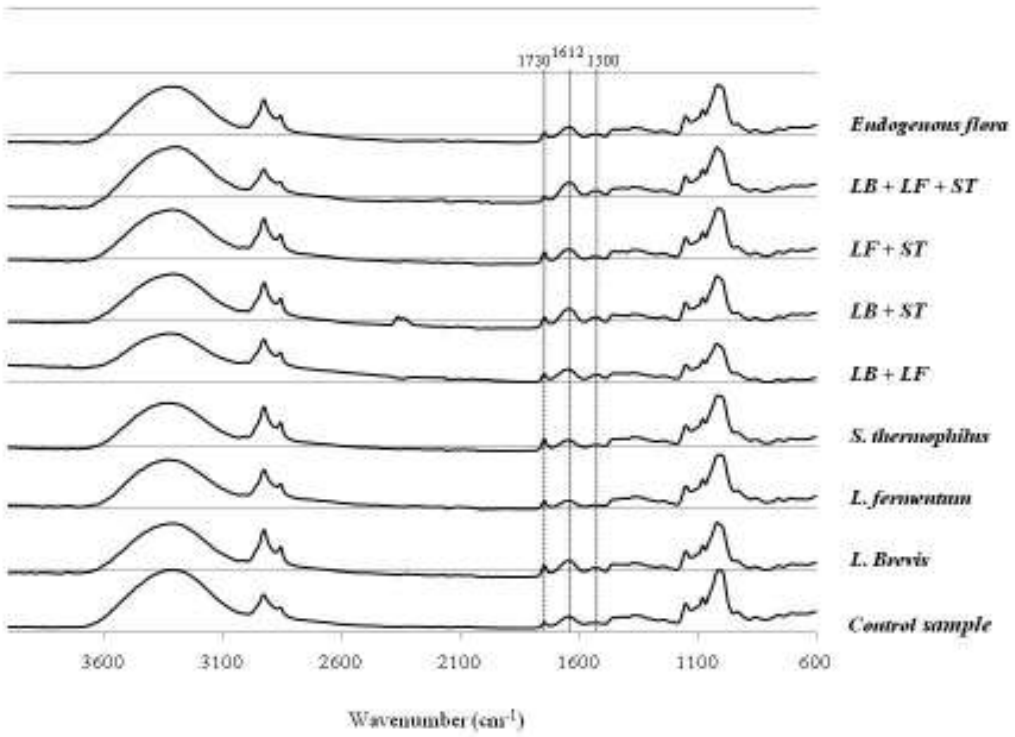
Soluble sugars provide a ready carbon source for microorganisms' production of organic acids. As a result, a decrease in reducing sugars was observed when samples were fermented with *Lactobacillus fermentum* (LF), *Streptococcus thermophilus* (ST), LB+ST, LF+ST, LB+LF+ST and endogenous microflora. A different trend on reducing sugars was found in samples fermented with *Lactobacillus brevis* and LB+LF where an increase was observed.

Table 1 show that fermentation with *Lactobacillus brevis* and the LB+LF mixture promoted a decrease of, respectively, 35% and 21% in starch content. This decrease is a result of the amylolitic activity of the inoculums. Microbial amylases of these species were able to hydrolyze starch chains leading to its solubilization, which explains the increase in reducing and total soluble sugars observed for these samples (Table 1).

Differences between starch content of control sample and the others fermented samples found by the enzymatic method were considered not significant. However, FT-IR spectra of samples insoluble fractions showed that all the tested inoculums promoted changes in starch content as it is possible to detect by modifications in bands corresponding to carbohydrates around 1000 cm^{-1} (Figure 1 a and b). This modifications are particularly noticed in samples fermented with *L. brevis* and LB + LF, which is consistent with the enzymatic analysis. Besides the general transformation in carbohydrates bands, there was a specific decrease in the starch peak at 991 cm^{-1} of samples fermented with *L. brevis*, LB + LF, LB + ST, LB + LF + ST and endogenous flora (Figure 1b). According to Correia et al. (2004), this could be related to a decrease in amylose content due to the starch hydrolysis. In fact, the hypothesis of starch hydrolysis explains the concomitant increase in total soluble sugars in fermented samples prepared with LB + ST and LB + LF + ST (Table 1).

FT-IR spectra of insoluble constituents of samples (Figure 1a) also revealed an increase in peaks at 1612 and 1500 cm^{-1}, assigned to proteins (amide I and amide II respectively) in samples fermented with *L. brevis*, LB + LF, LB + ST, LF + ST, LB + LF + ST and endogenous flora. The increase of insoluble protein content was reported to be a result of loss in insoluble matter content, through hydrolysis of other flour constituents by the action of microbial enzymes, and/or by the contribution of cells of fermenting microorganisms (Chavan and Kadam, 1989; Shayo et al., 2001). Our results showed that the accumulation of insoluble protein in these samples was at least partially caused by the observed decrease in starch content. In samples fermented with *L. fermentum* and *S. thermophilus* the decrease in proteins bands is more evident. One can conclude that these strains promoted a stronger proteolysis which overlaps the effect of insoluble protein concentration.

a)



b)

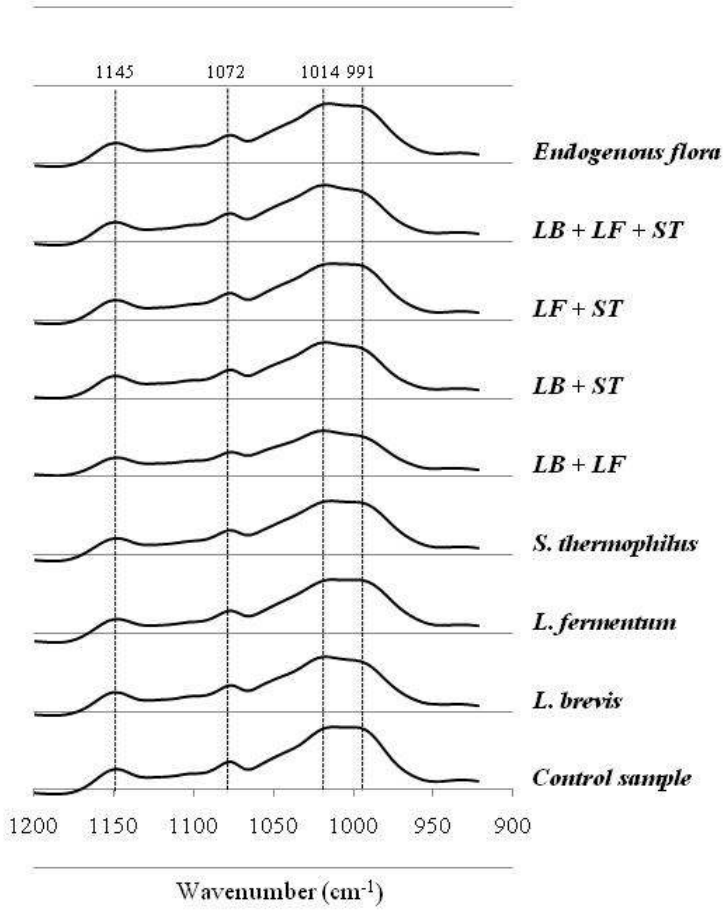


Figure 1 – **(a)** FT-IR spectra of control sample and samples fermented with *L. Brevis*, *L. Fermentum*, *S. Thermophilus*, *L. brevis* + *L. fermentum* (LB+LF), *L. brevis* + *S. thermophilus* (LB+ST), *L. fermentum* + *S. thermophilus* and *L. brevis* + *L. fermentum* + *S. thermophilus* (LB+LF+ST). **(b)** Spectral sub-region (1200 to 900 cm^{-1}).

Total protein increased in all fermented samples (Table 1). This increase results from dry matter loss by the conversion of non-proteic compounds, such as starch and other flour constituents, which were converted to volatile compounds. However, according to Asiedu et al.(1993), as in most human diets protein is more limiting than carbohydrates, any process that increases protein content (even if it occurs at the expense of carbohydrates) is nutritionally

advantageous. The addition of inoculum also contributes to the increase of total protein by the supply of cellular proteins.

The microorganisms are able to hydrolyze proteins into usable amino acids and peptides. Additionally, during their growth cycle, they can synthesize amino acids from metabolic intermediates (Au and Fields, 1981). As a consequence, an increase in soluble proteins and amino acids can occur. This explains the increase of 24% and 57% observed in soluble protein and free amino acid contents, respectively, of sample fermented with *Lactobacillus brevis* (Table 1). In contrast, soluble protein decreased in the other fermented samples. Soluble proteins of these samples were hydrolyzed to amino acids by bacterial proteases and peptidases.

Amino acids can be also consumed during their metabolic activity. When the amino acids increase (promoted by both the above mentioned synthesis and proteins hydrolysis) is higher than the amino acids intake, it is observed a global increase in amino acid levels. This is the case of samples fermented with *L. brevis*, *S. thermophilus*, LB+LF and endogenous flora, where it was observed an increase in free amino acid levels. When samples are fermented with *L. fermentum* or with mixed cultures of *S. thermophilus*, a decrease in amino acid levels takes place, indicating amino acids consumption by these inoculums (Table 1).

As a consequence of protein and/or starch hydrolysis by microbial enzymes, all fermented samples presented a higher *in vitro* protein digestibility values in comparison with control sample (Table 1). In case of traditional fermentation, protein digestibility increased from 20 to 52%. The increase in protein digestibility has been reported for some sorghum traditional fermented foods (Axtell et al., 1981; Chavan et al., 1988; Graham et al., 1986; Hassan and El Tinay, 1995; Moneim et al., 1995; Osman, 2004). The conjugation of the three studied species

(LB+LF+ST) an IVPD value of 65%, even higher than traditional fermentation, was obtained. We can conclude that conjugation of these lactic acid strains promoted symbiotic effects on protein quality leading to an IVPD improvement.

Figure 2 presents electropherograms of insoluble prolamins, which according to Nunes et al. (2004), show high molecular weight (HMW) aggregates, 66kDa trimer, 45 kDa dimer and γ, α, β monomers. The optical densities of each one of these electrophoretic spots of fermented samples were determined and expressed as a fraction of α and γ spot of control sample (Table 2).

As we can see from optical densities in Table 2, there was a decrease in spots correspondent to HMW aggregates, α and γ, β, 45 kDa and 66 kDa prolamins for almost all fermented samples in comparison to control sample. The exception is for β spot of sample fermented with LB+LF, LB+ST and LF+ST and α and γ spot of sample fermented with LB+LF for which there was an increase in optical densities. The observed decrease in the remaining spots of prolamins indicates that insoluble prolamins were hydrolyzed by bacterial proteases. We can conclude that all the used strains present proteolytic activities.

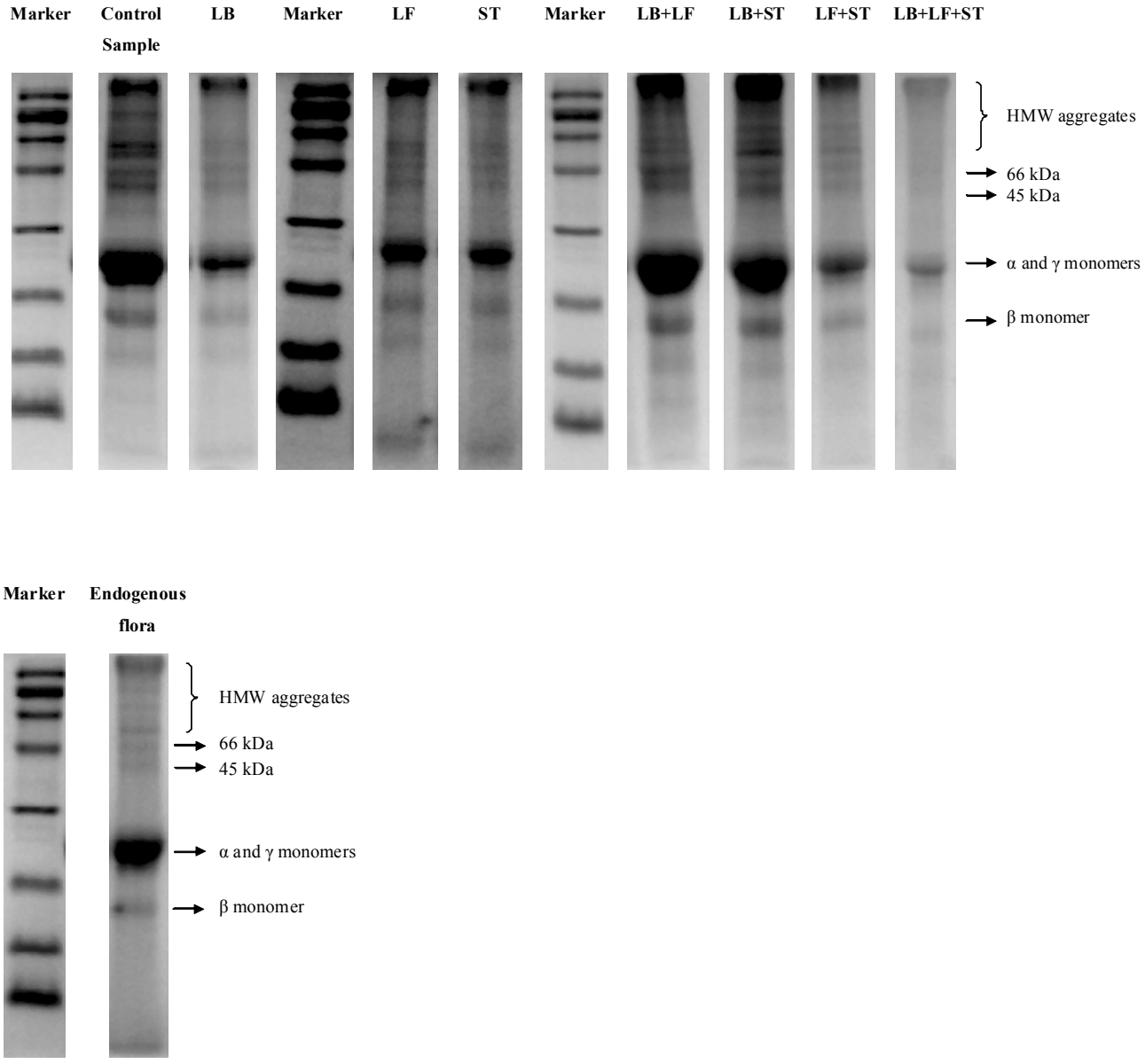


Figure 2 – Electrophoretic gels of insoluble proteins extracted from control sample and samples fermented with *L. brevis*, *L. fermentum*, *S. thermophilus*, *L. brevis* + *L. fermentum* (LB+LF), *L. brevis* + *S. thermophilus* (LB+ST), *L. fermentum* + *S. thermophilus* and *L. brevis* + *L. fermentum* + *S. thermophilus* (LB+LF+ST).

A possible explanation for the increase of some spots of LB+LF, LB+ST and LF+ST is related to the cleavage of S-S bonds of HMW aggregates and was previously reported by Nunes et al. (2005). According to these authors, when S-S bonds of HMW aggregates are cleaved, the oligomers are disrupted and their constituents are revealed leading to an increase of α, γ or β monomers in these samples. Furthermore, in the case of samples fermented with LB+LF, LB+ST, it seems that proteins are more concentrated in the insoluble fraction of these samples (Figure 2). As stated before, there was a consumption of starch and non starch components by these inoculums, leading to a protein concentration on the insoluble fraction of these samples and in consequence to the increase of the spots. Although a consumption of starch and/or non starch components was also observed on the other samples, the extension of insoluble proteins hydrolysis and its conversion into microbial proteins is superior to the concentration effect, thus this effect on protein concentration is not observable.

Samples fermented with LB+LF+ST presented the lower optical densities for all electrophoretic spots (Table 2). As a result of fermentation with this inoculum, optical density of β monomer spot dropped to a value below the detectable limit. These pronounced decreases on all electrophoretic spots of this sample are related to the strong proteolytic capacity obtained though the symbiosis of *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*. According to Nunes et al. (2004b), the un-reducible 45 kDa proteins are associated with the decrease on sorghum digestibility with cooking. The higher decrease in the 45 kDa oligomers content of sample fermented with LB+LF+ST could be responsible for its high IVPD value.

Table 2 – Optical densities of electrophoretic spots of α and γ monomers, β monomers, 45 and 66 kDa oligómeros and HMW of insoluble proteins extracted from control and fermented samples

Samples	α and γ monomers [1]	β monomer [1]	45 kDa oligomer [1]	66 kDa oligomer [1]	HMW aggregates [1]	Total
Control Sample	1.00	0.25	0.27	0.27	1.06	2.85
L. brevis	0.40	0.19	0.21	0.22	0.91	1.93
L. fermentum	0.50	0.20	0.21	0.21	0.95	2.07
S. thermophilus	0.47	0.19	0.19	0.19	0.86	1.90
LB+LF	1.06	0.39	0.24	0.25	0.90	2.84
LB+ST	0.93	0.39	0.24	0.24	1.03	2.83
LF+ST	0.37	0.29	0.19	0.20	0.99	2.04
LB+LF+ST	0.30	0.00	0.15	0.16	0.42	1.03
Endogenous Flora	0.53	0.21	0.17	0.17	0.48	1.56

[1] Optical densities of spots expressed as a fraction of the higher control sample optical density (spot of α and γ monomers of control sample).

The comparison between protein profiles of fermented samples and control samples could be another way to predict the improvement of protein digestibility with fermentation. Table 3 presents the decrease in electrophoretic spots promoted by fermentation, expressed as a percentage of control sample spots. These values are given by the difference between the sum

of control sample spots and the sum of fermented samples spots. As we can see on Table 3, with exception of samples fermented with LB + LF and LB+ST, the decrease of electrophoretic spots optical densities follow the same trend as the IVPD values. These values reflect the first parcel of the formula of IVPD determination. The discrepancy verified in samples fermented with LB + LF and LB+ST are due to the above mentioned effect of protein concentration. Even with this discrepancy there is a correlation between the percentage of decrease of electrophoretic spots and the percentage of IVPD (r = 0.83).

Table 3 –Percentage of decrease of total optical densities of electrophoretic spots of insoluble proteins of fermented samples (as a function of control sample) and IVPD values.

Samples	Decrease of electrophoretic spots optical densities (%)	IVPD (%)
L. brevis	32.3	39.2
L. fermentum	27.4	36.7
S. thermophilus	33.3	46.5
LB+LF	0.4	31.4
LB+ST	0.7	41.3
LF+ST	28.4	34.6
LB+LF+ST	63.9	64.8
Endogenous Flora	45.7	52.4

4. Conclusions

All the tested inoculums composed by lactic acid bacteria strains are able to fermented sorghum flour. The most effective inoculum was found to be composed by a mixed culture of *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*. With this inoculum, a high IVPD value was attained by the conjugation of the strong amylolitic activity of *L. brevis* and the strong proteolytic activity of *L. fermentum* and *S. thermophilus*.

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6. AVALIAÇÃO DOS EFEITOS PROMOVIDOS PELA UTILIZAÇÃO CONJUNTA DE BACTÉRIAS LÁCTICAS E LEVEDURAS E PELA ADIÇÃO DE MALTE DE SORGO

6.1. Materiais e métodos

Revitalização microrganismos e preparação dos isolados

As estirpes microbianas utilizadas neste trabalho foram adquiridas à empresa DSMZ. As bactérias lácticas foram adquiridas na forma de liofilizados e as leveduras na forma de cultura em meio sólido.

Por altura da utilização das bactérias lácticas na secção 4 deste capítulo, promoveu-se a revitalização de todas as espécies microbianas (bactérias e leveduras), de acordo com o procedimento descrito anteriormente, e preparação e congelamento dos respectivos isolados microbianos para as utilizações futuras. Para tal, em tubos tipo *eppendorf* estéreis, foram adicionados 100 µL de glicerol a 200 µL de cada estirpe microbiana. As misturas foram conservadas em arca frigorífica a -70ºC até a sua utilização.

Preparação dos inóculos

Os inóculos utilizados nas fermentações com as espécies *Lactobacillus brevis, Lactobacillus fermentum*, *Streptococcus thermophilus*, *Issatchenkia orientalis* e *Saccharomyces cerevisiae* foram preparados a partir dos respectivos isolados congelados.

Os isolados das respectivas espécies a utilizar foram transferidos para meios de cultura líquidos (MRS Broth, Merck) e incubados à temperatura de crescimento em estufa com agitação (37 ºC para *L. fermentum* e *S. thermophilus*, 30 ºC para *Lactobacillus brevis* e 25 ºC para *I. orientalis* e *S. cerevisiae*). Após sucessivas repicagens para meio de cultura líquido, os microrganismos foram repicados para meio sólido (MRS agar, Merck). O pH de ambos os meios foi acertado para 6,2-6,5 previamente à sua esterilização em autoclave (Uniclave 88, AMJ) a 121ºC durante 20 minutos.

Cada uma das espécies foi incubada à respectiva temperatura óptima até atingirem a sua fase exponencial de crescimento (aproximadamente 24 horas). Nesta fase, foram preparadas

suspensões para cada uma das diferentes espécies microbianas em soro fisiológico. As suspensões foram preparadas de acordo com o procedimento descrito nas secções 4 e 5.

Todas as operações foram realizadas em câmara de fluxo laminar (Braun Horizontal, BBH4) na proximidade do bico de Bunsen de modo a garantir as condições de assepsia.

Maltagem do sorgo

Para a preparação de malte de sorgo os grãos foram manualmente lavados, demolhados e estendidos durante cinco dias em papel de filtro sobre tabuleiros de alumínio previamente esterilizados, de acordo com o procedimento descrito na secção 5 do capítulo 3. Os grãos germinados foram secos em estufa de vazio e, após remoção das radículas, moídos num moinho de café e peneirados para a obtenção de uma granulometria de 0,3 mm.

Fermentação com *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus* com adição prévia de sorgo maltado

Das análises químicas e espectroscópicas realizadas a todas as amostras fermentadas, foi possível concluir que o inóculo mais efectivo na melhoria da qualidade nutricional do sorgo seria o constituído pelas espécies *Lactobacillus brevis, Lactobacillus fermentum e Streptococcus thermophilus* em simultâneo. Optou-se assim por testar o efeito da adição de sorgo maltado previamente à fermentação com as respectivas espécies.

Para tal, a 15 g de farinha de sorgo, em Erlenmeyer esterilizado e rolhado, foi adicionada água numa proporção 1:10 (m/v). A mistura foi cozida durante 10 minutos para promover a gelatinização do amido. Após arrefecimento à temperatura ambiente até atingir 35ºC, foram adicionados 7,5 g de malte e 75 mL de água destilada. A mistura foi incubada durante 30 minutos de modo a promover a actuação das enzimas endógenas do grão sobre os constituintes da farinha. Posteriormente a amostra foi fervida por mais 5 minutos para a inactivação das enzimas.

A amostra foi, por fim, autoclavada e fermentada de acordo com o descrito para a fermentação com as espécies *Lactobacillus brevis, Lactobacillus fermentum e Streptococcus thermophilus* em simultâneo.

Fermentação do sorgo *com Lactobacillus brevis, Lactobacillus fermentum e Streptococcus thermophilus* com adição de levedura

Para avaliar os efeitos da utilização de leveduras associadas às bactérias lácticas, duas espécies de leveduras foram seleccionadas: *Issatchenkia orientalis* e *Saccharomyces cerevisiae*.

Neste estudo foram realizadas duas fermentações em paralelo em que, para além das bactérias *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus*, numa das fermentações foi utilizada a levedura *Issatchenkia orientalis*, e na outra foi utilizada a levedura *Saccharomyces cerevisiae*.

As amostras foram preparadas e fermentadas como descrito para a fermentação em que apenas actuavam as três espécies bacterianas em simultâneo, apenas diferindo na adição de levedura. De modo a manter o volume total de inóculo igual aos ensaios anteriores (5 mL), foram adicionados às amostras 1,25 mL de suspensão de cada espécie.

Preparação das amostras e análises químicas

A preparação das amostras, bem como as análises químicas (determinação de aminoácidos livres, proteína solúvel e total, açúcares redutores e totais solúveis, amido e digestibilidade proteica *in vitro*), foram realizadas de acordo com o descrito nas secções anteriores deste capítulo.

Acompanhamento do crescimento microbiano das espécies em meio de cultura

De modo a prever qual (ou quais) as espécies predominantes nas fermentações com inóculos mistos, promoveu-se ainda o acompanhamento do crescimento em meio de cultura das bactérias *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus* e das leveduras *Issatchenkia orientalis* e *Saccharomyces cerevisiae*, isoladas ou nas respectivas combinações utilizadas nas fermentações.

Para tal, 10 mL de meio de cultura líquido (MRS Broth, Merck) foram adicionados à tubos de ensaio rolhados. Para estudar o crescimento conjunto das três espécies bacterianas, promoveu-se a inoculação de três tubos com 100 µL de suspensão bacteriana de cada espécie, preparadas

como descrito anteriormente. Para o acompanhamento do crescimento das bactérias em conjugação com as leveduras, outros seis tubos foram inoculados com 75 μL de cada uma das suspensões bacterianas, sendo que em três deles foram também adicionados 75 μL de suspensão de *Issatchenkia orientalis* e, nos outros três, 75 μL de suspensão de *Saccharomyces cerevisiae*. Foram ainda inoculados 3 tubos com 300 μL de cada espécie isolada. Os tubos de ensaio foram incubados durante 5 dias As temperaturas de incubação foram as utilizadas nas fermentações com os respectivos inóculos. Posteriormente, uma alíquota de cada tubo foi transferida para meio MRS sólido (MRS Broth, Merck). As colónias resultantes (células viáveis que sobreviveram ao crescimento em simbiose) foram removidas dos meios de cultura com o auxílio de uma ansa e analisadas por FT-IR. Os respectivos espectros foram submetidos à Análise em Componentes Principais (PCA).

6.2. Resultados e discussão

Em todas as amostras fermentadas, verifica-se uma diminuição no pH como resultado da produção de ácidos orgânicos pelos microrganismos. Segundo Mugula *et al.* (2003c), a adição de malte à farinha promove uma descida mais rápida do pH. Como resultado, verifica-se um menor valor de pH para amostra em que se procedeu à adição de malte previamente à fermentação.

Tal como se havia verificado nas secções anteriores, a proteína total sofre um aumento em todas as amostras fermentadas como resultado da sua concentração no meio por diminuição de outros constituintes da farinha. É de referir que, uma vez que a determinação de proteína total é feita através da determinação de azoto total, a hidrólise de proteína insolúvel em proteína solúvel e aminoácidos ou a sua conversão em metabolitos azotados nunca se reflecte nos valores de proteína total, dado que o teor de azoto total mantém-se constante nestas situações. Uma diminuição no teor de proteína total indica assim uma perda de azoto por evaporação, por exemplo, pela conversão de aminoácidos (arginina) em amoníaco. De igual modo, a produção de proteína resultante da actividade metabólica dos microrganismos, não promove variações no teor de azoto total uma vez que resulta da conversão de azoto da farinha em azoto de proteína microbiana. Assim sendo, um aumento no teor de proteína total é sempre aparente e

derivado da sua concentração na amostra por perda de matéria-seca. Durante o processo fermentativo, os microrganismos podem promover o consumo de outros componentes não azotados da farinha e a sua conversão em compostos voláteis que se libertam durante a liofilização, originando uma diminuição na matéria-seca. A adição do inóculo também poderá contribuir para a proteína total pelo acréscimo de proteína celular bacteriana.

Na amostra em que se promoveu adição prévia de malte, verifica-se um aumento nos aminoácidos livres e na proteína solúvel, comparativamente à amostra inoculada apenas com LAB´s e à amostra não fermentada (Tabela 2). Este aumento é indicativo de ataque às proteínas insolúveis, resultando na solubilização de aminoácidos e proteínas em quantidades superiores às necessidades de consumo dos microrganismos. A adição de malte fornece um sistema proteolítico que permite a degradação de proteínas e a sua conversão em pequenos peptídeos e aminoácidos.

Verifica-se ainda um aumento nos açúcares redutores e totais solúveis para a mesma amostra. A diminuição no teor de amido foi de 99,5% e deve-se ao ataque ao amido por amilases endógenas, fornecendo assim açúcares livres disponíveis para utilização como fonte de energia para as bactérias lácticas inoculadas posteriormente.

A hidrólise do amido, e a consequente solubilização de açúcares, ocorre numa extensão maior no caso da amostra incubada com sorgo maltado do que nas restantes amostras. Este extenso ataque ao amido apresenta, contudo, algumas desvantagens nutricionais, uma vez que se traduz num aumento do índice glicémico do produto final obtido.

Seria de esperar que das alterações promovidas no teor de aminoácidos e proteína solúvel (indicativas de ataque às proteínas insolúveis) e do extenso ataque ao amido, resultasse uma maior acessibilidade da pepsina às proteínas da amostra com adição de malte, o que se reflectiria num aumento da digestibilidade proteica. No entanto, o valor de digestibilidade proteica diminui de 65 para 31% com a adição de malte. Este resultado pode ser consequência desta amostra possuir uma razão proteína de sorgo/proteína microbiana inferior à amostra fermentada sem adição de malte. Ao incubarmos a farinha com malte, estamos a enriquecê-la em aminoácidos e peptídeos resultantes da hidrólise proteica promovida pelas proteases

endógenas. Desta forma, estamos a fornecer amino-azoto livre que pode ser directamente utilizado pelos microrganismos favorecendo assim o seu crescimento e, consequentemente, aumentando o teor de proteína microbiana. Assim, para além da dimunuição da proteína do sorgo promovida pela fermentação, temos a diminuição originada pela germinação. Nas condições em que a determinação da digestibilidade é efectuada, a pepsina não parece clivar microrganismos, apenas prolaminas. Testes realizados com pepsina e microrganismos não demonstraram a produção de aminoácidos. Ao efectuarmos o ensaio de digestibilidade proteica na fracção insolúvel da amostra, a % de proteína digerida por pepsina será menor porque haverá uma menor quantidade de proteína de sorgo e uma maior proporção de proteína microbiana. Uma vez que a proteína intracelular não é clivada por pepsina, obtém-se um menor valor de IVPD (31%).

Tabela 2 – Determinação do pH, aminoácidos livres, proteína solúvel, proteína total, digestibilidade proteica, açúcares redutores, açúcares totais solúveis e amido total da testemunha e das amostras fermentadas.

Amostras	pH	Aminoácidos Livres (mg/g)[1,2]	Proteína Solúvel (mg/g)[1,2]	Proteína Total (%)[1,2]	IVPD (%)[1,2]	Açúcares Redutores (mg/g)[1,2]	Açúcares Totais (mg/g)[1,2]	Amido Total (%)[1]
Testemunha	6,41	4,79 ± 0,36	4,35 ± 0,17	9,07 ± 0,15	19,66 ± 0,49	18,81 ± 0,59	96,73 ± 0,55	64,03 ± 2,97
LB+LF+ST	4,29	4,05 ± 0,16	1,21 ± 0,08	13,30 ± 0,78	64,75 ± 0,51	9,93 ± 0,46	124,14 ± 1,25	65,88 ± 0,88
LB+LF+ST + Malte	2, 97	6,65 ± 0,20	9,91 ± 0,06	11,67 ± 0,16	31,37 ± 0,51	247,87 ± 2,30	675,01 ± 1,98	0,34 [2] ± 0,19
LB+LF+ST +IO	4,41	8,16 ± 1,87	8,15 ± 0,05	11,72 ± 0,46	31,14 ± 0,69	20,81 ± 0,80	230,10 ± 5,25	39,07 [2] ± 1,52
LB+LF+ST +SC	4,74	6,51 ± 0,03	9,56 ± 0,48	11,40 ± 1,33	37,41 ± 0,52	20,11 ± 1,26	247,71 ± 13,22	34,50 [2] ± 1,78

[1] Média de três réplicas ± desvio-padrão.
[2] Diferenças em relação à testemunha consideradas significativas para $p < 0.05$.

O facto da digestibilidade proteica *in vitro* diminuir com a adição de malte, pode não significar uma diminuição da disponibilidade proteica *in vivo*. Isto por que, numa digestão *in vivo*, participam outras enzimas para além da pepsina. O sistema enzimático mais complexo presente no nosso organismo apresenta capacidade para digerir alguns microrganismos, o que pode significar que a conversão de proteína de sorgo em proteína microbiana não tem necessariamente que resultar numa menor digestibilidade proteica *in vivo*, podendo ser um factor positivo em termos nutricionais.

No caso das amostras fermentadas conjuntamente com *I. orientalis* e *S. cerevisiae*, o teor de amido diminuiu para 39 e 35%, respectivamente, comparativamente à amostra fermentada apenas com bactérias. Esta diminuição é resultado da actividade de amilases microbianas que clivam o amido em dextrinas e açúcares livres que passam para a fase solúvel da amostra. Este facto justifica o aumento paralelo no teor de açúcares redutores e totais solúveis (Tabela 2).

Tal como a adição de malte, a adição de leveduras ao inóculo promoveu uma diminuição da digestibilidade proteica. Na tabela 2, verifica-se que a introdução de *I. orientalis* e *S.cerevisiae* promoveu uma diminuição dos valores de digestibilidade de 65 para 31 e 37%, respectivamente. Este resultado pode estar relacionado com o facto de, apesar da concentração de microrganismos totais ser a mesma nos dois inóculos, a concentração de cada uma das espécies de bactérias lácticas é menor no inóculo com leveduras. Este resultado é consistente com o maior teor de proteína solúvel e aminoácidos verificado para esta amostra comparativamente à amostra fermentada apenas com bactérias (Tabela 2). O maior teor em peptídeos e aminoácidos poderá apenas significar que há uma menor utilização destes compostos com este inóculo.

As secções 4 e 5 deste capítulo revelaram que as três espécies de bactérias lácticas utilizadas, sobretudo a espécie *S. thermophilus*, apresentam uma elevada capacidade proteolítica. O mesmo parece não se verificar com as leveduras. A espécie *S. cerevisiae*, de acordo Hough (1985), não apresenta proteases extracelulares não possuindo assim capacidade de utilizar proteínas. Deste modo, o facto de estarmos a enriquecer o inóculo numa espécie pouco proteolítica em detrimento de outras mais proteolíticas poderá explicar a diminuição da

digestibilidade verificada nas fermentações com o inóculos composto por bactérias lácticas e leveduras.

A análise das espécies predominantes nas fermentações poderá explicar os resultados químicos obtidos.

De acordo com o diagrama de coordenadas factoriais da Figura 4, as colónias resultantes da inoculação do meio de cultura com *L.brevis*, *L. fermentum* e *S. thermophilus* (LB+LF+ST) encontram-se no quadrante (PC1(-), PC2 (-), quadrante oposto ao quadrante no qual se situa as colónias provenientes do meio inoculado com *L. brevis* (PC1(+), PC2 (+)). As colónias (LB+LF+ST) partilham ainda o PC1 negativo com as colónias de *S. thermophilus*, e o PC2 negativo com as colónias de *L. fermentum*. Estes resultados podem ser interpretados como uma predominância das espécies *L. fermentum* e *S. thermophilus* sobre o *L. brevis* após os cinco dias de incubação das três espécies bacterianas em meio de cultura.

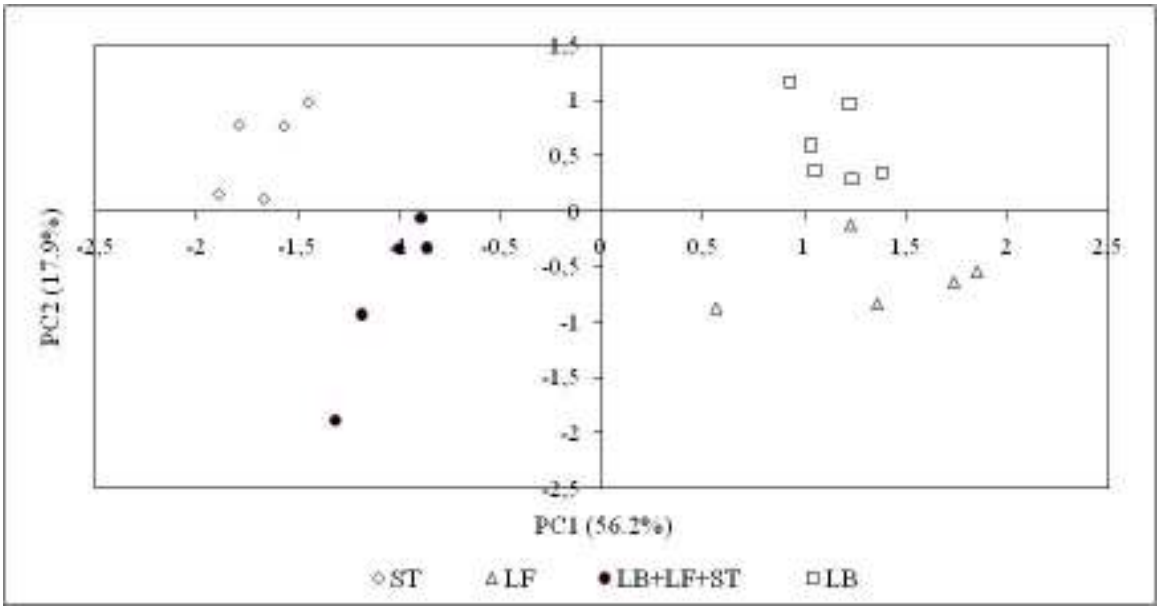

Figura 4: Diagrama de coordenadas factoriais de PC1 *vs* PC2 das colónias viáveis presentes em meio de cultura após inoculação com *L. brevis* (LB), *L. fermentum* (LF), *S. thermophilus* (ST) e *L. brevis* + *L. fermentum* + *S. thermophilus* (LB+LF+ST), e incubação por cinco dias.

Ao inocularmos o meio de cultura com as bactérias *L.brevis*, *L. fermentum*, *S. thermophilus* e com a levedura *I. orientalis*, verifica-se que após cinco dias de incubação as células viáveis resultantes encontram-se agrupadas no mesmo quadrante das colónias de *L. fermentum* (PC1 (-) e PC2 (+)) (Figura 5). Parece haver uma predominância desta espécie nestas colónias. Por outro lado, as colónias provenientes do meio com levedura e bactérias encontram-se ainda agrupadas com as colónias de *L. brevis* no PC2(+). O eixo PC2 parece estar a promover a separação entre as colónias da levedura *I. orientalis* e as colónias de bactérias lácticas.

Se ao invés de *I. orientalis* adicionarmos *S. cerevisiae* às bactérias lácticas, as células víaveis provenientes da mistura dos 4 microrganismos encontram-se, mais uma vez, no mesmo quadrante das colónias de *L. fermentum* (PC1 (+) e PC3 (+)) e agrupadas com *L. brevis* no PC3 (+) (Figura 6). Encontram-se, ainda, agrupadas com as colónias de *S. cerevisiae* no PC1 (+) e no quadrante oposto ao das colónias de *S. thermophilus* (PC1 (-) e PC3 (-)). Estes resultados poderão significar uma menor predominância da espécie *S. thermophilus* em detrimento da espécie *S. cerevisiae*.

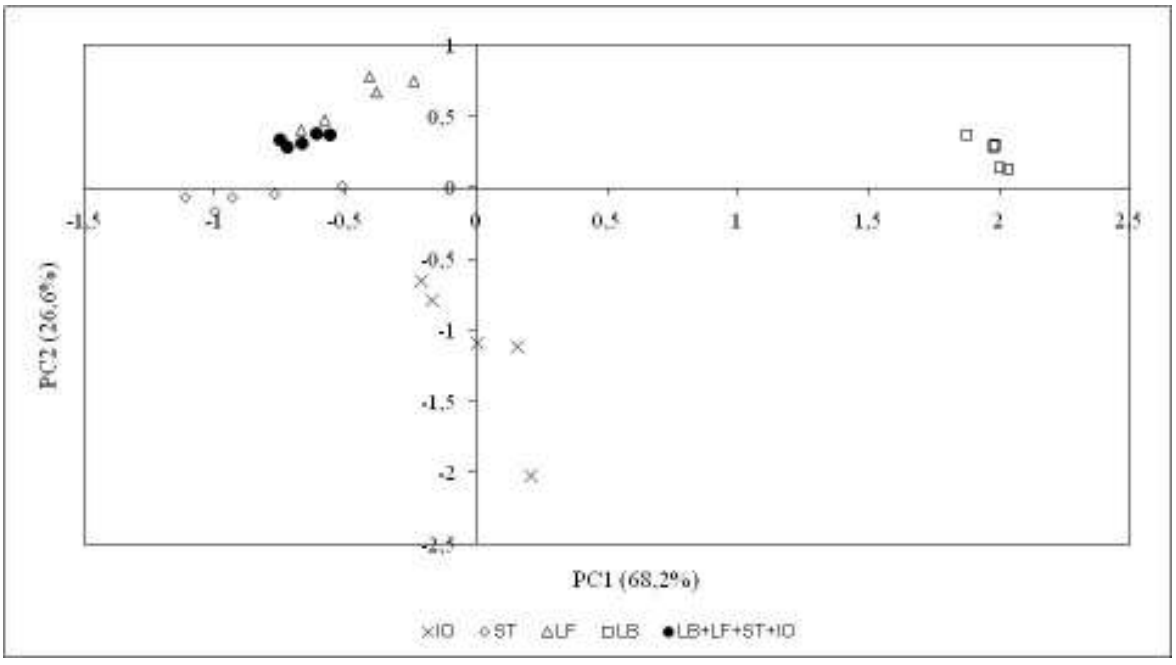

Figura 5: Diagrama de coordenadas factoriais de PC1 *vs* PC2 das colónias viáveis presentes em meio de cultura após inoculação com *L. brevis* (LB), *L. fermentum* (LF), *S. thermophilus*

(ST), *I. orientalis* (IO), *L. brevis* + *L. fermentum* + *S. thermophilus* (LB+LF+ST) e *L. brevis* + *L. fermentum* + *S. thermophilus* + *I. orientalis* (LB+LF+ST+IO) e incubação por cinco dias.

Em suma, conjugação das espécies bacterianas com ou sem adição de leveduras resulta sempre numa simbiose favorável à espécie *L. fermentum*, espécie que se mantém como uma das predominantes nos três tipos de inóculos. A conjugação de bactérias com leveduras não parece resultar numa simbiose favorável às leveduras, eventualmente por estas terem sido adicionadas numa proporção de 1 para 3 em relação às bactérias.

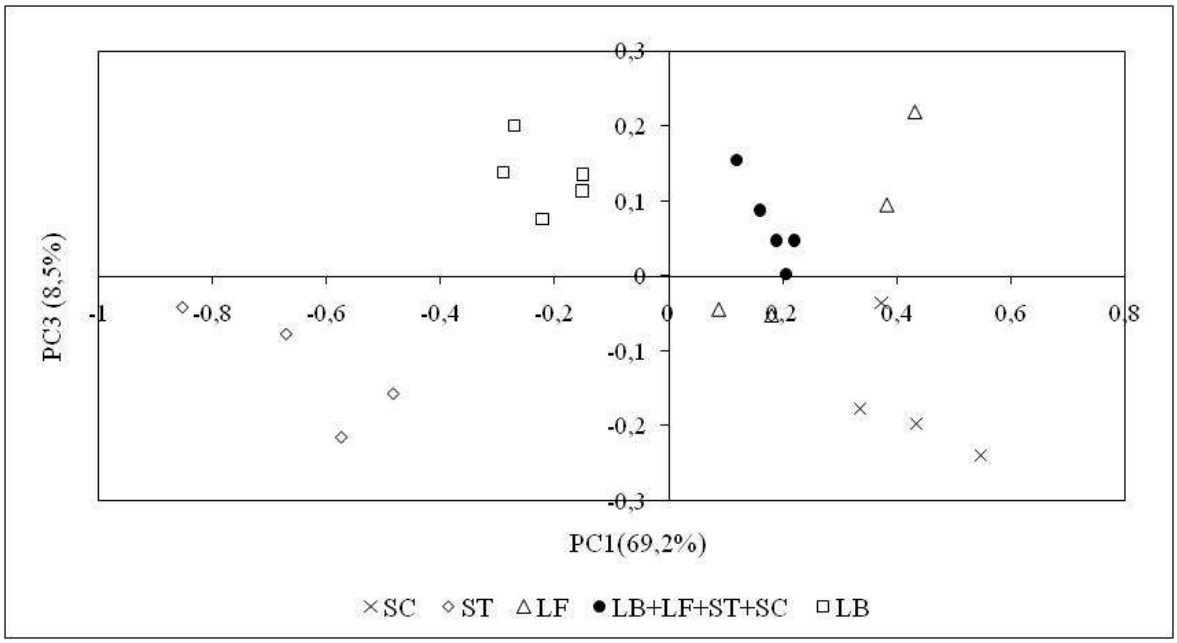

Figura 6: Diagrama de coordenadas factoriais de PC1 *vs* PC2 das colónias viáveis presentes em meio de cultura após inoculação com *L. brevis* (LB), *L. fermentum* (LF), *S. thermophilus* (ST), *S. cerevisiae* (SC), *L. brevis* + *L. fermentum* + *S. thermophilus* (LB+LF+ST) e *L. brevis* + *L. fermentum* + *S. thermophilus* + *S.cerevisiae* (LB+LF+ST+SC) e incubação por cinco dias.

Deste estudo pode-se que concluir que a conjugação das bactérias *L.brevis*, *L. fermentum* e *S. thermophilus* com as leveduras *I. orientalis* e *S. cerevisiae* não se mostrou vantajosa tendo originado uma diminuição nos valores de digestibilidade proteica. Essa diminuição nos valores de IVPD deve-se, por um lado, ao facto de estarmos a enriquecer o inóculo numa espécie

menos proteolítica em detrimento de outras mais proteolíticas e, por outro lado, ao facto do crescimento da espécie introduzida não ser muito favorecido.

A utilização de malte permitiu a hidrólise de proteínas e amido em compostos azotados e açúcares mais simples e solúveis pela introdução de enzimas hidrolíticas ao sistema, o que poderá resultar num aumento da digestibilidade proteica *in vivo* (apesar da diminuição verificada para a digestibilidade proteica *in vitro*). A hidrólise do amido, contudo, ocorre numa extensão muito elevada, o que se reflecte numa desvantagem em termos nutricionais. Tal como referido no capítulo 1, a existência de amido resistente no sorgo é vantajosa, sobretudo para os habitantes dos países de consumo de sorgo para os quais este cereal é a principal fonte de energia. A presença de amido resistente traz benefícios a saúde, como por exemplo na prevenção da diabetes e do cancro do cólon e no aumento da saciedade. O extenso ataque ao amido promovido pela adição de malte diminui, contudo, o teor de amido. Ao ser clivado pelas amilases endógenas, o amido foi convertido na sua quase totalidade em açúcares solúveis. Essa conversão se traduz numa maior velocidade de entrada dos açúcares na corrente sanguínea e, consequentemente, num aumento do índice glicémico do produto resultante.

7. FERMENTAÇÃO DO SORGO COM A FLORA MICROBIANA DOS GRÃOS DE KEFIR

"Sorghum fermentation with Kefir grains microflora"

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Sorghum fermentation with Kefir grains microflora

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Key words: *Sorghum bicolor* (L.) Moench, Kefir, fermentation, weaning food.

Abbreviations Used

ATR - Attenuated Ttotal Reflection

DNS - 3,5- Dinitrosalicylic acid

FT-IR – Fourier Transform Infrared

HMW –High Molecular Weight

1H NMR- Nuclear magnetic resonance

IVPD – *In vitro* protein digestibility

OD – Optical density

PCA – Principal Components Analysis

SDS-PAGE – Sodium Dodecyl Sulfate Polycrylamide Gel Electrophoresis

SNV - Standard Normal Variate

ABSTRACT

Sorghum flour was fermented with the microflora of Kefir grains in order to evaluate the application of these species on the production of sorghum fermented foods. Protein carbohydrates and amino acids were analyzed to assess nutritional quality of the final product.

The results revealed that fermentation promoted an improvement on sorghum nutritional quality. The analysis by Fourier Transform Infrared spectroscopy (FT-IR) show that the microorganisms promoted the hydrolysis of starch, resulting in a decrease of 96.3% in starch content. Protein analysis by SDS-PAGE revealed that 33.68% of the insoluble protein was hydrolyzed with fermentation. As a result of starch and protein hydrolysis, there was an increase of 72.53% in protein digestibility. A better amino acids balance was also obtained with fermentation.

With this study, we conclude that kefir grains may represent an alternative starter culture for sorghum fermentation. With this inoculum, easily accessible to the population, it is possible to obtain a nutritionally improved and safety sorghum product.

Introduction

In semi-arid regions of the world, due to the limited supply of animal foods, people diet is based mainly on cereals. Among cereals, sorghum (*Sorghum bicolor* (L.) Moench) is one of the five most important in world food supply. Millions of people in African and Asia rely on sorghum as a major or principal source of dietary protein and energy (Graham et al., 1986). However, due to poor quality of sorghum proteins, low starch availability and presence of certain anti-nutritional factors, the use of sorghum as human food is confined to world's poorest people.

To overcome these nutritional constrains of sorghum foods, and also by cultural reasons, sorghum is usually fermented to produce thick porridges for adults or liquid gruels for infants. Natural fermentation of sorghum meals increases protein digestibility (Hassan and El Tinay, 1995) and protein content (Chavan et al., 1988), provides a better essential amino acid composition (Au and Fields, 1981), makes the starch more available (Kazanas and Fields, 1981), decreases the tannin content (Hassan and El Tinay, 1995), and increases the vitamin content (El Tinay et al., 1979).

The increasing urbanization leads to an increase in the request for traditional foods in a way that traditional methods of supplying such foods are no longer applied. As a result, household fermentation technologies have been converted to an industrial scale in some African countries in order to provide value added products that meet urban population demand for traditional products (Belton and Taylor, 2004; Gadaga et al., 1999). There is, therefore, a need to replace a traditional fermentation to a controlled process with pure starter cultures to obtain nutritionally improved products of consistent quality, safety and stability. However, there are

no commercially available starter cultures for small-scale production of sorghum fermented foods.

Kefir grains are a natural mixed culture used for centuries in the Caucasus area for the production of the traditional milk drink through lactic acid and alcoholic fermentations (Plessas et al., 2005). The Kefir grains are white or lightly yellowish irregular masses with a cauliflower-like structure in which microorganisms are contained in a matrix of proteins and polissacharides (Bosch et al., 2006).

Many microorganisms have been isolated from Kefir grains, including yeasts (*Kluyveromyces*, *Candida*, *Torulopsis* and *Sacharomyces sp*), lactobacilli (*L. brevis*, *L. acidophilus*, *L. casei*, *L. helveticus*, *L. delbruecki*, *L. kefir*, *L. plantarum*, *L. fermentum*), streptococci (*Streptococcus salivarius*), lactococci (*Lc. Lactis ssp. thermophilus*, *Leuconostoc mesenteroides* and *L. cremoris*) and occasionally acetic acid bacteria (Garrote et al., 2001; Pintado et al., 1996; Simova et al., 2002). A symbiotic relationship exists between the microbes present in the Kefir grains and it has been shown that there are specific species that always occur in the grains. (Pintado et al., 1996).

In this study, sorghum flour was fermented with the microflora of Kefir grains. The purpose of this work was to evaluate the application of these species on the improvement of sorghum nutritional quality.

2. Materials and methods

2.1. Sorghum flour

Sorghum grains, of the Australian variety Jumbo, were purchased in a retail trade and ground with a coffee mill to pass through a 3×10^{-4} m sieve.

2.2. Flour fermentations

For fermentation with Kefir grains, a sample of sorghum flour (0.015 kg) was mixed with sterilized water (1:10 w/v), in sealed E-flasks. This mixture was boiled for 1 minute under vigorous stirring in order to homogenize the gelatinization process prior the autoclavation. The sample was then autoclaved at 121 ºC for 15 min. After cooled, at room temperature (*ca.* 25ºC), the sample was inoculated with 0.008 kg of Kefir grains of artisan origin (Portugal). This sample was incubated for five days at ambient temperature (*ca.* 25ºC). After this period of time, kefir grains were removed from the sample.

An unfermented sample (control sample) was prepared as described for controlled fermentations, omitting the inoculation step.

2.3. Preparation of samples

The pH of samples was measured and each of the samples was divided in two portions. The first one was freeze dried and ground again. This portion was kept to determine total protein, soluble proteins, reducing sugars, total sugars and amino acids. Total protein was determined directly on the freeze dried powder. To analyse each of following soluble components; soluble proteins, reducing sugars, total sugars and amino acids; 1 x 10^{-3} kg of the freeze dried samples were mixed with distillated water in a proportion of 1:20 w/v, and magnetic stirred during 1 hour. Samples were centrifuged (2500 x *g* at room temperature) for 3 minutes and the analytes determined in the supernatants. In the case of soluble protein, pH was adjusted to 2 (with HCl) after water addition.

The second portion of each sample was centrifuged at 24000 x *g* during 20 minutes (Sigma 3K30 centrifuge - Osterode am Harz, Germany) and the residues were freeze dried and ground

again. These residues were used on the determination of total starch, FT-IR analysis, *in vitro* protein digestibility (IVPD) assay and SDS-PAGE analysis of insoluble proteins.

2.4. pH measurement

The pH of the samples was measured with a glass electrode.

2.5. Reducing sugars determination

Reducing sugars were determined by the 3,5- dinitrosalicylic acid (DNS) colorimetric method, with glucose as the standard (Miller, 1959). 1 x 10^{-3} L of DNS reagent was added to 1 x 10^{-3} L of sample supernatant. The mix was kept in a boiling water bath for 5 minutes. After cooling to room temperature (*ca.* 25ºC) in a cold water bath, 1 x 10^{-2} L of distillated water was added. The absorbance at 540 nm was measured (Shimadzu UV-160A spectrophotometer), interpolating the value obtained with calculated values for glucose solutions of known concentration. The blanks were prepared by substituting sample solution for distilled water.

2.6. Total sugars determination

A modified phenol-sulphuric acid method was used to determine total sugars present in the samples (Dubois et al., 1956). 1 x 10^{-3} L of 5% phenol was added to 1 x 10^{-4} L of sample. Then, 1 x 10^{-3} L of concentrated sulfuric acid was added and the mixture was kept in a boiling water bath for 10 minutes. After cooling to room temperature (*ca.* 25ºC), in a cold water bath, the absorbance at 490 nm was measured (Shimadzu UV-160A spectrophotometer - Tokyo, Japan). The amount of sugars was then determined by reference to a standard curve prepared with glucose. The blanks were prepared by substituting sample solution for distilled water.

2.7. Soluble proteins determination

Soluble proteins were determined with a TCA concentration - BCA assay protocol kit for protein determination (Sigma – Missouri, USA).

2.8. Free amino acids determination

The quantitative measurement of free amino acids was made using the ninhydrin reaction (Plummer, 1978). 2 x 10^{-3} L of buffered ninhydrin reagent (8 x 10^{-4} kg of ninhydrin and 12 x 10^{-5} kg of hydrindantin dissolved in 3 x 10^{-2} L of 2-methoxyethanol plus 1 x 10^{-2} L of acetate buffer 4 M, pH 5.5) were added to 2 x 10^{-3} L of sample and heated in a boiling water bath for 15 minutes. The mixture was cooled to room temperature (*ca.* 25ºC), 3 x 10^{-3} L of 50% ethanol was added and the absorbance was read at 570 nm after 10 minutes (Shimadzu UV-160A spectrophotometer). The amount of amino acids was determined by reference to a standard curve previously prepared with arginine. The blanks were prepared by substituting sample solution for distilled water.

2.9. Total amino acids analysis

For the analysis of total amino acids, samples were submitted to acid hydrolysis with 6 M HCl during 24 h according to procedure described by Zumwalt (1987). The acid was removed by centrifugal evaporation at 40 ºC under vacuum (Univapo 100 H, Uniequip, Munich, Germany) and the residue was dissolved in 1 x 10^{-3} L of 0.1 M HCl. After filtration through a 45 x 10^{-6} m membrane, amino acids were derivatizated as described by MacKenzie (1987). Amino acids were determined by GC-FID as heptafluorobutylisobutyl derivatives, using norleucine as internal standard. A Clarus 400 gas chromatography apparatus (Perkin Elmer) with a flame ionisation detector (FID) and equipped with a 30 m length DB-1 column (J&W Scientific, Folsom, CA, USA) with i.d 25 x 10^{-5} m and 1 x 10^{-7} m film thickness was used. The

oven temperature program used was: initial temperature 70 ºC during 1 minute, a rise in temperature at a rate of 2 ºC min^{-1} until 170 ºC and then a rate of 16 ºC min^{-1} until 250 ºC and this temperature was maintained for 5 minutes. The injector and detector temperatures were, respectively, 250 and 260 ºC.

2.10. Total proteins determination

Control and fermented samples were submitted to determination of total N by elementary analysis. The percentage of protein was determined by multiplying for 6.25.

2.11. Total starch Determination

The amount of total starch was determined using a total starch determination kit (Megazyme International Ireland Limited- Wicklow, Ireland).

2.12. Fourier Transform Infrared spectroscopy

Insoluble constituent of control and fermented samples were analyzed by Fourier Transform Infrared (FT-IR) spectroscopy. The FT-IR spectra were obtained using a Golden Gate single reflection diamond attenuated total reflection (ATR) system in a Bruker IFS-55 spectrometer (Ettlingen, Germany). The spectra were recorded in absorbance mode from 4000 to 500 (cm^{-1}), co-adding 128 scans at 8 (cm^{-1}) resolution. Five replicates were collected for each sample. The spectra obtained were transferred into a data analysis package (Barros, 1999). For Principal Component Analysis (PCA) (Jolliffe, 1986), the 1780-800 (cm^{-1}) region was selected as it comprises lipids (1743 (cm^{-1})), protein (1650 – 1500 (cm^{-1})) and starch signals (around 1000 (cm^{-1})), major components of sorghum flour. Each spectrum was SNV corrected (Standard Normal Variate). The PCA allowed the characterization of the sample relationships (scores plans) and the recovery of their sub-spectral profiles (loadings).

2.13. in vitro protein digestibility assay

Control and fermented samples were subjected to *in vitro* protein digestibility assay (IVPD) using pepsin (Sigma - P-7000 - 975 U/(mg) protein) as described by Nunes, Correia, Barros and Delgadillo (2004a), with some modifications. Flour samples (1×10^{-1} kg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 M KH_2PO_4 pH 2 buffer) in a water bath (37^oC) for 0 (t0) and 120 minutes (t120). After this period of time, the digestions were stopped by the addition of 1×10^{-4} L of 2 M NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, room temperature) for 3 minutes and the supernatants discarded. The residues were washed with 1×10^{-3} L of 0.1 M K_2HPO_4 pH 7 buffer, centrifuged and washed again with 1×10^{-3} L of water. These residues, with insoluble undigested proteins, were freeze-dried and weighed. The content of total N that remains in control samples (t0) and in samples after 120 minutes (t120) of protein digestion was determined by elementary analysis of N.

The proportion (as a function of initial total protein) of soluble proteic material prior to pepsin addition was calculated by the sum of soluble protein and amino acids values.

The percentage of insoluble proteins digested by pepsin was calculated by the difference between insoluble protein content at 0 (t0) and 120 (t120) minutes of digestion. The *in vitro* protein digestibility was defined as the sum of protein solubilized by endogenous and microbial enzymes and the proportion of insoluble protein digested by pepsin through the follow formula:

$$\text{IVPD} = \left(\frac{\text{SP} + \text{AA}}{\text{TP}} + \frac{\text{Protein content (t0)} - \text{Protein content (t120)}}{\text{Protein content (t0)}} \right) \text{x } 100$$

Where SP is the soluble proteins content prior pepsin digestion; AA is the free aminoacids content prior pepsin digestion; TP is the total protein; Protein content (t0) – Protein content (t120) is the content of proteins solubilized by pepsin in the IVPD assay and Protein content (t0) in the content of insoluble proteins prior IVPD assay

2.14. SDS-PAGE analysis

To study undigested proteins of the IVPD assay, residues of 0 and 120 minutes of pepsin digestion were submitted to 2 hour protein extraction with 5 x 10^{-4} L 0.0125M $Na_2B_4O_7.10H_2O$ (pH10), 2% (m/v) SDS and 1% (v/v) 2-mercaptoethanol (Hamaker, 1995) to analyze its non-digestible proteins. After extraction, the mixtures were centrifuged (2500 x g, room temperature) for 3 minutes. Supernatants, with extracted proteins were prepared for SDS-PAGE by mixing 2 x 10^{-5} L of the protein extract with 1 x 10^{-5} L of SDS-PAGE sample buffer [2% (w/v) SDS, 0.0625 M Tris, 10% (v/v) glycerol, 0.01% (w/v) bromophenol blue, pH 6.8]. The samples were heated, for 5 minutes, in a boiling water bath and 7 x 10^{-6} L were applied in a 15% acrylamide SDS-PAGE gel (Laemmli method). The gels were run in a Mini-Protean II electrophoretic cell with Power Pac 300 (Bio-Rad - Hercules, USA). Electrophoresis was conducted at 170 V for 1.5 h until the tracking dye, bromophenol blue, reached the bottom of the resolving gel. Molecular weight markers were used to compare electrophoretic mobility of proteins (Broad Range Markers – BioRad). Gels were stained with Coomassie Blue R (GE Healthcare - Uppsala, Sweden) and destained with 40% methanol and 10% acetic acid (Shewry et al., 1995).

2.15. Analysis of SDS-PAGE images

For image analysis each gel image was acquired using the calibrated imaging Gel-Doc (Bio-Rad - Hercules, USA) and analyzed with the Quantity One v4.6 software (Bio-Rad- Hercules,

USA). The software allowed background subtraction, automatic band detection and comparative analysis of normalised band optical densities (ODs).

2.16. ^{1}H NMR

High resolution nuclear magnetic resonance (NMR) spectroscopy was used to evaluate the effects of fermentation on the chemical composition of the supernatants. The samples were prepared to contain 10% D_2O, used as the internal lock, and 0.02% sodium 3-(trimethylsilyl) propionate-d_4 (TSP-d_4), used as chemical shift and intensity reference, their pH adjusted to 4 and transferred to 5 x 10^{-3} m o.d. NMR tubes. The ^{1}H 1D NMR spectra were recorded at 27ºC on a Bruker Avance DRX-500 spectrometer, operating at 500.13 MHz for proton, using the 'noesypr1d' pulse sequence: RD–90º–t_1–90º–t_m–90º–acquire FID (Bruker library), where RD is the relaxation delay (5.0 s), t_1 represents the first increment in a NOESY experiment (3 μs), and t_m is the mixing period (100 ms). The water signal was suppressed by presaturation during the relaxation delay and mixing time. Each ^{1}H 1D spectrum consisted of 128 scans of 32K data points with a spectral width of 8012.82 Hz and an acquisition time of 2.04 s. Prior to Fourier transformation (FT), the free induction decays (FIDs) were zero-filled to 32k points and multiplied by an exponential line-broadening function of 0.3 Hz. The 1D spectra were manually phased, baseline corrected and the chemical shifts referenced to the TSP resonance to 0 ppm.

2.17. Statistical analysis

All values are expressed as means and standard deviation for three replicates, with exception of those obtained by SDS-PAGE analysis. Mean values of treatments were compared by Student's *t* test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

When sorghum is fermented with Kefir grains microflora, pH drops from 6.41 to 3.78 as a result of the production of organic acids from sugars (Table 1). The production of organic acids is supported by the ^{1}H NMR spectrum (Figure 1) which shows the drastic increase of lactic, formic, acetic, butyric, isobutyric and succinic acids in fermented sample (Figure 1b). Similar results were obtained on a previous work on sorghum fermentation (Correia et al., 2004).

Table 1 - Chemical analysis results of the control sample and sample fermented with Kefir grains microflora.

Samples	pH	Free Amino acids (mg/g)[1,2]	Soluble Protein (mg/g)[1,2]	Total Protein (%)[1,2]	IVPD (%)[1,2]	Reducing Sugars (mg/g)[1,2]	Total Soluble Sugars (mg/g)[1,2]	Total Starch (%)[1,2]
Control Sample	6.41	4.79 ± 0.36	4.35 ± 0.17	9.07 ± 0.15	19.66 ± 0.49	18.81 ± 0.59	96.73 ± 0.55	64.03 ± 2.97
Fermented Sample	3.78	4.19 ± 0.03	25.64 ± 0.62	13.14 ± 0.60	33.92 ± 0.47	356.07 ± 0.95	641.64 ± 1.17	2.36 ± 0.01

[1] Mean of three replicates ± standard deviation.
[2] Differences from control sample were considered significant at $p < 0.05$.

a)

b)

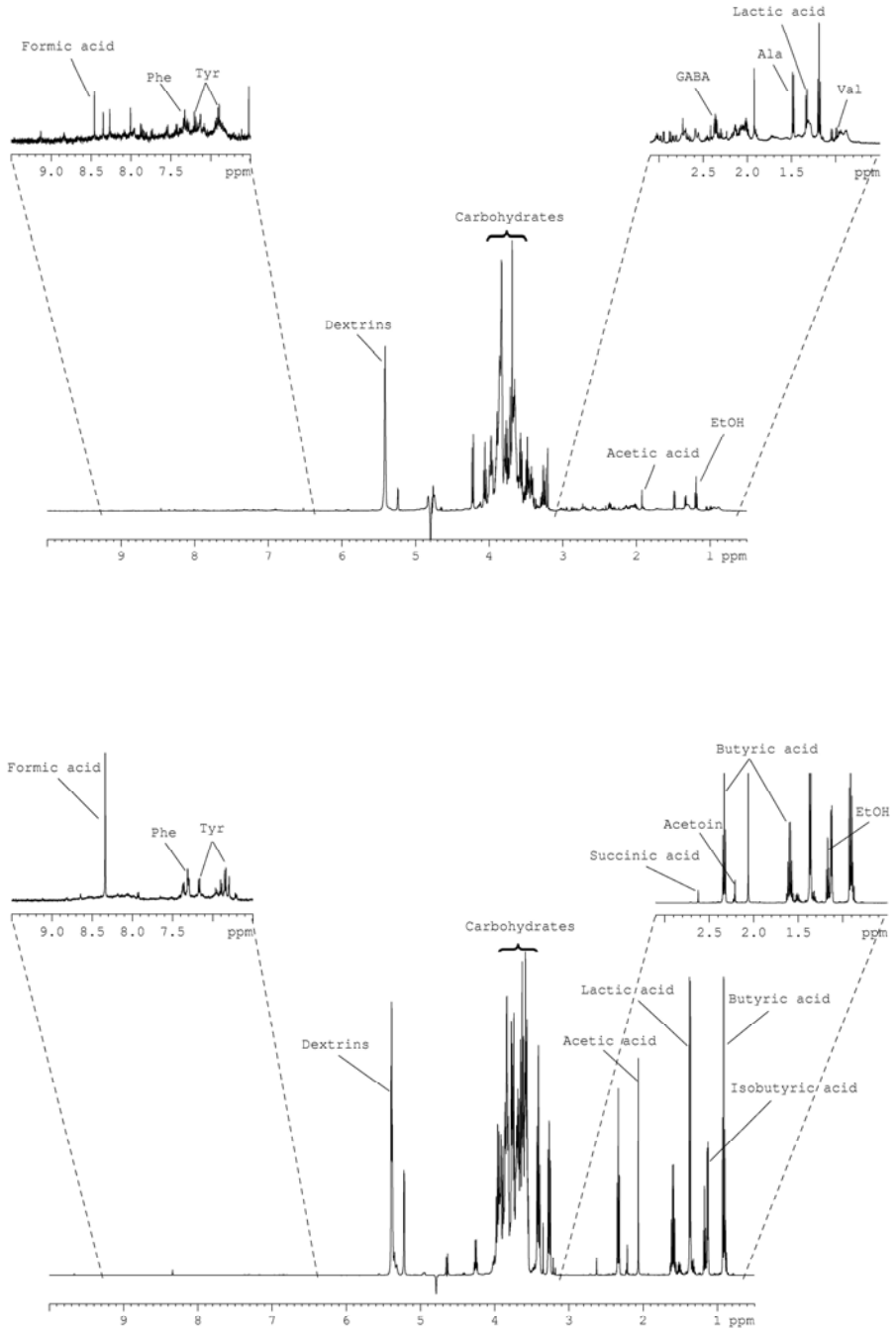
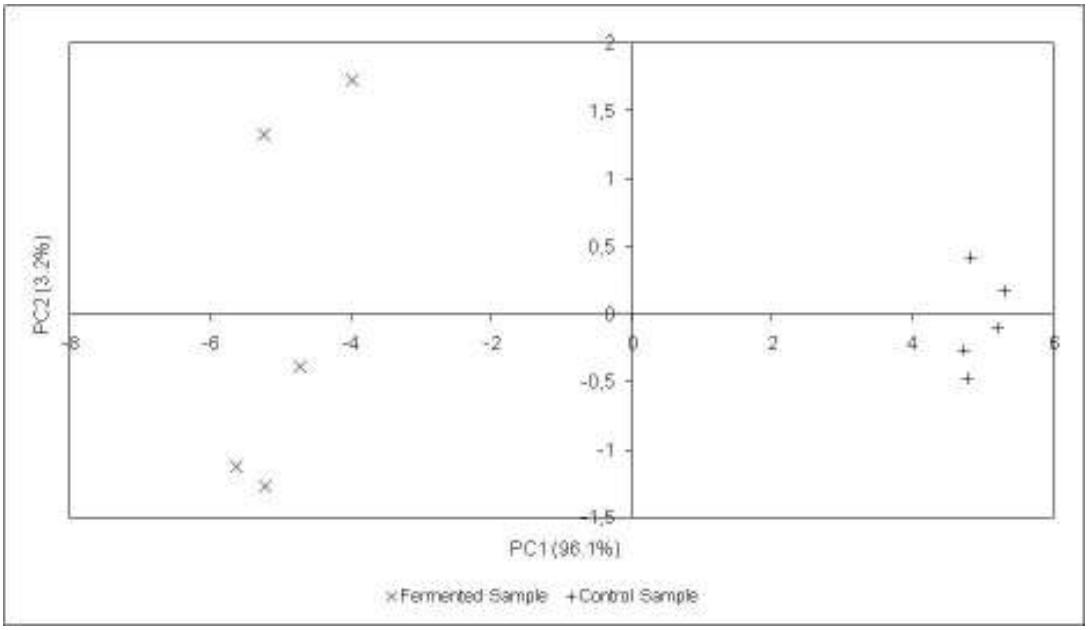


Figure 1 - ^{1}H NMR spectra of **(a)** control sample and **(b)** sorghum fermented with Kefir grains microflora.

Kefir grains microflora presents a strong amylolytic activity, as we can see on Table 1 by changes on carbohydrates contents. Cleavage of starch chains into ready utilizable sugars lead to a decrease of total starch from 64.03 to 2.36% and, consequently, an increase of reducing and total soluble sugars from 18.81 and 96.73% to 356.07 and 641.64%, respectively (Table 1). The ^{1}H NMR spectrum of the control sample (Figure 1a) show a relatively broad signal at 5.4 (ppm), arising from the H1 protons from the glucose anomeric carbon involved in $\alpha(1\rightarrow4)$ glycosidic linkages of dextrins and signals between 3.2 and 4.2 (ppm) from dextrins (Figure 1). This indicates that part of the starch was solubilized by cooking, originating visible signals in the spectrum. With the fermentation process, all of these signals increase as a result of starch hydrolysis by microbial amylases which promoted more dextrins to pass into the liquid phase (Figure 1b).

The decrease of starch contents is also perceptible by the multivariate analysis of FT-IR spectra (Figure 2). The scores scatter plot of samples (Figure 2a) shows that PC1 axis provides a distinguishing between control sample (PC1 (+)) and sample fermented with Kefir grains microflora (PC1(-)). PC1 loading profile (Figure 2b) shows that control sample is characterized by a sign arising from starch at 933 (cm^{-1}), which confirms its superior starch content determined by the chemical analysis. Fermented sample presents a higher contribution of proteins manifested by bands with typical signs at 1647, 1624 (amide I) and 1523 (cm^{-1}) (amide II). Once again, this suggests an accumulation of insoluble protein in the fermented sample provided by the decrease in starch content. Similar results were obtained on a previous work on traditional fermented sorghum (Correia et al., 2004).

a)



b)

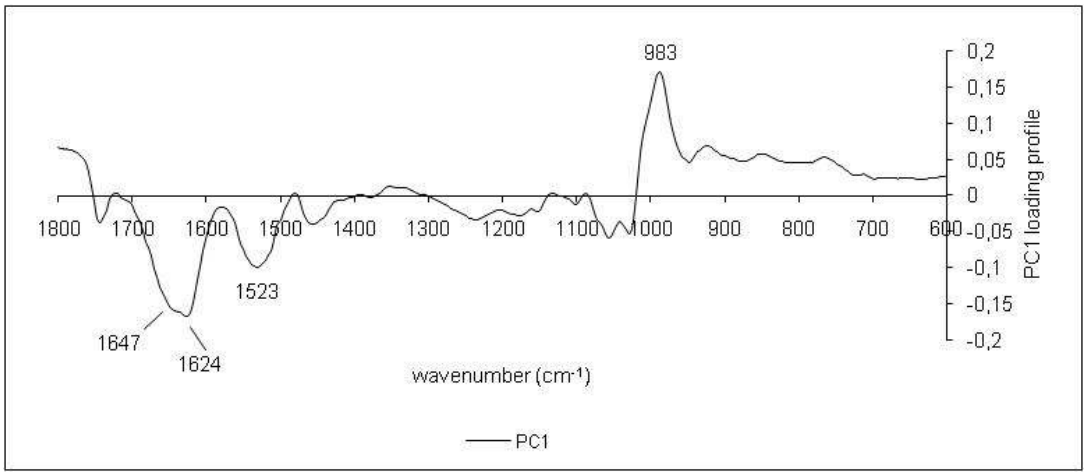


Figure 2 – (a) PCA scores scatter plot (PC1 *vs.* PC2) and **(b)** PC1 Loadings of the FT-IR spectra of control sample and samples fermented with Kefir grains microflora.

Total proteins suffer an increase from 9.07 to 13.14% with fermentation (Table 1). The increase in total protein is an apparent increase which results from the loss in the dry matter content, through hydrolysis of starch and/or other flour constituents and its conversion on volatile compounds liberated during lyophilization.

Free amino acids content suffers a decrease of 12.52% with fermentation as a result of its utilization by the microflora during their metabolic activity (Table 1). NMR analysis of soluble fraction reflects a consumption of alanina, valine and γ-amino-butyric acid. On the other hand, an increase in phenylalanine and tyrosine occurs (Figure 1a and b). The decrease in γ-amino-butyric acid and increase in phenylalanine and tyrosine were already observed on sorghum fermented with *Lactobacillus fermentum* (Correia et al., 2004).

Insoluble proteins were hydrolyzed into more simple and soluble products, resulting on a strong increase in soluble proteins, from 4.35 to 25.64% (Table 1). SDS-PAGE of insoluble proteins gives us information about the extension of this hydrolysis and the composition of hydrolyzed proteins.

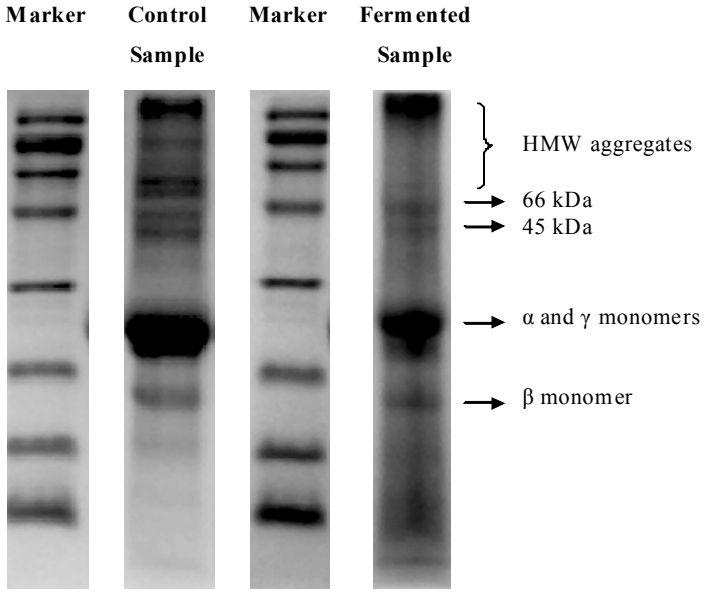


Figure 3 - Electrophoretic gels of insoluble proteins extracted from control sample and samples fermented with Kefir grains microflora.

Electrophoregrams of insoluble proteins extracted from samples residues are presented in Figure 3. Sorghum prolamins were well characterized previously by Nunes et al. (2004b) which allowed the identification of spots that correspond to high molecular weight (HMW) aggregates, 66kDa trimmer, 45 kDa dimmer and γ, α, β monomers. As we can see on Table 2, there was a decrease from 2.85 to 1.89 on the optical densities of these electrophoretic spots. This result reveals that 33.68% of the insoluble proteins were hydrolyzed by the microorganisms. The higher decrease in optical densities were observed on spots of high molecular weight aggregates and γ and α monomers (48.00 and 40.57%, respectively). We can conclude that these fractions were the principal target of proteolysis.

Table 2 – Optical densities of electrophoretic spots of α and γ monomers, β monomers, 45 and 66 kDa oligómeros and HMW, extracted from of insoluble proteins of control sample and sample fermented with Kefir grains microflora and total optical densities.

Samples	**α and γ monomers** [1]	**β monomer** [1]	**45 kDa oligomer** [1]	**66 kDa oligomer** [1]	**HMW aggregates** [1]	**Total**
Control Sample	1.00	0,25	0.27	0.27	1.06	2.85
Fermented Sample	0.52	0.26	0.24	0.24	0.63	1.89

[1] Optical densities of spots expressed as a fraction of the higher control sample optical density (spot of α and γ monomers of control sample).

Fermentation with Kefir grains microflora lead to an enhancement of 72.53% on the *in vitro* protein digestibility of sorghum flour (Table 1). Amylolytic and proteolytic activities promoted by microbial enzymes make proteins more accessible to pepsin attack, thus enhancing its digestibility.

Table 3 – Amino acids analysis of control sample and sample fermented with Kefir grains microflora.

Amino acids	*Control Sample (%)* [1]	*Fermented Sample (%)* [1]
Alanine	8.6 ± 0.3	8.1 ± 0.3
Glycine	3.3 ± 0.1	2.7 ± 0.0 [2]
Valine	9.0 ± 0. 2	11.2 ± 0.1 [2]
Threonine	1.9 ± 0. 1	2.4 ± 0.1 [2]
Serine	6.3 ± 0.1	7.2 ± 0.1 [2]
Leucine	25.8 ± 0.1	22.7 ± 0.2 [2]
Isoleucine	1.9 ± 0.1	2.7 ± 0.1 [2]
Proline	8.5 ± 0.3	9.1 ± 0.1 [2]
Methionine	0. 7 ± 0.1	0. 9 ± 0.0 [2]
Aspartic acid	6.9 ± 0.2	8.3 ± 0.3 [2]
Phenylalanine	4.0 ± 0.2	4.3 ± 0.0 [2]
Glutamic acid	14.8 ± 0.2	15.6 ± 0.4 [2]
Lysine	1.3 ± 0.1	1.3 ± 0.2
Tyrosine	2.6 ± 0.1	2.0 ± 0.1 [2]
Arginine	4.1 ± 0.2	1. 9 ± 0.1 [2]

[1] Mean of three replicates ± standard deviation.
[2] Differences from control sample were considered significant at $p < 0.05$.

Microorganisms can promote a *de novo* production of amino acids from metabolic intermediates during their growth cycle. As a consequence, proteins with different amino acids composition are obtained. Table 3 presents amino acids composition of samples. Apart from alanina and lysine (which doesn't change significantly), and from arginina, tyrosine, glycine and leucine (which decreases), all amino acids suffer an increase with fermentation (Table 3). The observed increase of the essential amino acids phenylalanine, isoleucina, methionine,

threonine and valine with fermentation provided a better amino acids balance, resulting on a protein quality enhancement. The observed decrease in arginina content with fermentation could be a result of its conversion to ammonia. As was said before, ^{1}H NMR spectra (Figure 1) show indeed the increase of phenylalanine and tyrosine in soluble fraction of the fermented sample.

Other changes revealed by ^{1}H NMR are the increase of ethanol and acetoin when comparing between the control and the fermented sample. These compounds were also found on other fermented products and result from the conversion of pyruvate by lactic acid bacteria (Kandler, 1983; Tsau et al., 1992).

4. Conclusions

We conclude that kefir grains may represent an effective, secure and easy accessible inoculum to be used as starter culture for sorghum fermentation.

In addition to improving the protein digestibility, the extensive starch attack promoted by kefir microflora is also nutritionally important because it results in a less viscous porridge without decreasing the nutrient and energy density.

In this particular case, the starch hydrolysis was very extensive, which could lead to the conclusion that might be disadvantageous due to the high glycemic index increase which would result. However, ^{1}H NMR spectrum of the fermented sample revealed the presence of a considerable amount of dextrins on the soluble fraction. This allows us to conclude that the increase in glycemic index would not be so marked.

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CAPÍTULO 5 – CARACTERIZAÇÃO FINAL DO ALIMENTO OBTIDO PELA FORMA DE PROCESSAMENTO PROPOSTA

1. INTRODUÇÃO

Tal como foi referido no capítulo 1, o baixo teor em aminoácidos essenciais, a baixa digestibilidade proteica e a baixa densidade energética são características inerentes ao sorgo que o tornam um alimento de baixa qualidade nutricional.

A acção dos microrganismos actuantes nos processos fermentativos permite, contudo, melhorar as características nutricionais dos alimentos a base de sorgo

No que respeita aos aminoácidos, estudos efectuados no *kisra* demonstraram um aumento no teor de aminoácidos essenciais e semi-essenciais, nomeadamente de metionina, valina, leucina, fenilalanina, treonina e tirosina (El Tinay *et al.*, 1979). A fermentação natural do sorgo durante cinco dias também revelou um aumento nos teores de lisina, leucina, isoleucina e metionina (Kazanas e Fields, 1981). Estudo realizados por Au e Fields (1981) confirmaram o incremento dos níveis de metionina e lisina no sorgo submetido a 4 dias de fermentação espontânea.

Para além da melhoria na digestibilidade proteica e do aumento no teor de proteínas solúveis e insolúveis, a fermentação promove ainda alterações a nível dos hidratos de carbono, resultando numa melhoria da digestibilidade do amido e diminuição do amido resistente. Estas

alterações devem-se à acção de amilases microbianas. À semelhança das amilases produzidas no processo germinativo, as amilases microbianas promovem a clivagem do amido em açúcares solúveis, o que permite liquefazer os alimentos amiláceos. Como consequência, é possível a obtenção de alimentos de viscosidade inferior, com as já referidas vantagens inerentes à maior densidade energética.

A fermentação promove ainda um incremento das características organolépticas e alterações na textura dos alimentos como resultado da formação de ácidos orgânicos, álcoois, aldeídos e outros produtos resultantes da actividade metabólica dos microrganismos.

2. OBJECTIVOS

Este capítulo teve como objectivo a caracterização do produto obtido pela fermentação do sorgo com as espécies *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus*.

Uma vez determinado o inóculo mais efectivo na melhoria da qualidade nutricional do sorgo, tornou-se importante proceder a uma caracterização mais exaustiva do alimento final resultante, nomeadamente no que respeita à qualidade proteica, à disponibilidade do amido e às características organolépticas.

Para tal, procedeu-se à caracterização do alimento final quanto à sua composição em aminoácidos, digestibilidade do amido, razão amilose/amilopectina, viscosidade e composição em compostos orgânicos voláteis.

3. ENQUADRAMENTO EXPERIMENTAL

Para além da digestibilidade proteica, a composição em aminoácidos, nomeadamente o teor em aminoácidos essenciais e o balanço em aminoácidos, é outro aspecto importante a ter em conta para a avaliação da qualidade proteica.

A determinação da composição total em aminoácidos foi efectuada por cromatografia em fase gasosa. A espectroscopia de Ressonância Magnética Nuclear de alta resolução de estado líquido (RMN), utilizada no acompanhamento da composição em componentes solúveis das amostras, permitiu a determinação dos aminoácidos solúveis.

Uma vez que o sorgo é a principal fonte de energia para as populações de consumo do sorgo, é importante avaliar a disponibilidade do amido no produto final. Como tal, foi realizado um ensaio de digestibilidade do amido e, uma vez que esta é influenciada pelo teor de amilose, promoveu-se ainda a determinação da razão amilose/amilopectina.

Quando se pensa no sorgo como matéria-prima para a preparação de alimentos complementares ao leite materno, a viscosidade torna-se uma característica especialmente importante. Contudo, os estudos sobre os efeitos da fermentação na viscosidade são escassos e os resultados são contraditórios, parecendo depender do tipo de microrganismos actuantes nas fermentações. Por essa razão, promoveu-se a determinação da viscosidade da papa obtida de modo a avaliar a possibilidade da sua utilização como alimento complementar de desmame.

As características organolépticas são outro aspecto a ter em conta, uma vez que a aceitação por parte dos consumidores está dependente das mesmas. É importante que o produto obtido pela fermentação controlada apresente características organolépticas semelhantes às dos produtos fermentados tradicionalmente. A determinação da composição em compostos orgânicos voláteis, nomeadamente dos ácidos orgânicos, aldeídos e álcoois formados na fermentação, e a sua comparação com os existentes na literatura para produtos fermentados tradicionalmente, pode constituir uma forma de aferir sobre as semelhanças organolépticas entre os dois tipos de produtos (de fermentação controlada e de fermentação tradicional).

A determinação dos compostos orgânicos voláteis foi realizada com recurso à micro-extracção em fase sólida (SPME). Os compostos voláteis adsorvidos foram separados e analisados por cromatografia em faes gasosa acoplada à espectrometria de massa (GC-MS). A determinação de alguns compostos orgânicos não voláteis, responsáveis pelas características organolépticas de alimentos fermentados tradicionalmente, foi possível pela análise dos espectros de RMN das fracções solúveis das amostras.

4. QUALIDADE PROTEICA, DIGESTIBILIDADE DO AMIDO, VISCOSIDADE E PEFI L VOLÁTIL DE SORGO FERMENTADO COM CULTURAS PURAS DE BACTÉRIAS LÁCTICAS

"Protein quality, starch availability, viscosity and volatile profile of a sorghum porridge fermented with commercial cultures of lactic acid bacteria"

Artigo científico submetido à revista "Journal of Cereal Science"

Protein quality, starch availability, viscosity and volatile profile of a sorghum porridge fermented with commercial cultures of lactic acid bacteria

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Keywords: Sorghum, fermentation, lactic acid bacteria.

Abbreviations Used

CFU – Cell Forming Units

DNS - 3,5- Dinitrosalicylic acid

^{1}H NMR- Nuclear magnetic resonance

HS-SPME - Headspace analysis by solid phase microextraction

IVPD – *In vitro* protein digestibility

IVSD - *In vitro* starch digestibility

LAB - Lactic acid bacteria

OD – Optical density

VOC - Volatile organic compound

ABSTRACT

Pure cultures of lactic acid bacteria were tested as inoculum for sorghum porridge fermentation. Sorghum cooked flour was inoculated with *Lactobacillus brevis* (DSM 6235), *Lactobacillus fermentum* (DSM 20052) and *Streptococcus thermophilus* (DSM 20617) and the protein quality and starch availability of the porridge were analyzed. As the bulk density is an important parameter for weaning foods, porridge viscosity was also carried out. Soluble constituents were analyzed by nuclear magnetic resonance (1H-NMR), and volatile organic compounds by headspace analysis using solid phase microextraction (HS-SPME) to evaluate organoleptic characteristics of the final porridge.

We conclude that the inoculum composed of *Lactobacillus brevis, Lactobacillus fermentum* and *Streptococcus thermophilus* could be used as starter for the production of the commercial weaning foods. Fermentation with this mixed inoculum increased sorghum nutritional quality rendering proteins and starch more digestible and increasing essential amino acids. Thinner sorghum porridge with the same nutritional density was obtained as a result of its viscosity decrease. The sorghum porridge obtained in this work presents several organic compounds usually found on traditional porridges.

1. Introduction

Sorghum porridges are prepared in many African countries for human consumption, and appear to be the most common type of food prepared from sorghum (Duodu et al., 2003).

In developing countries, the weaning process with sorghum porridges has been associated to undernourishment in human infants. Sorghum weaning foods have low energy and nutrient densities, and are a major cause of malnutrition among 6-24 month old children in sub-Saharan Africa (Onyango et al., 2004b). As these products are very thick, sorghum porridges are diluted to a flour concentration of 5-10 % to attain viscosities of less than 3000 cP to facilitate swallow. This concentration provide them an energy density (0.2 kcal/g) below the recommended 0.75 kcal/g provided by breast milk (Lorri and Svanberg, 1993; Mosha and Vicent, 2004; Westby and Gallat, 1991). The limited gastric capacity of infants and the low daily frequency of consumption means that these diluted sorghum porridges do not meet the infant's energy requirements (Traoré et al., 2004).

Besides low energy density, the poor nutritional quality of sorghum porridges is also attributed to its low starch availability (Hall et al., 1968), deficiency of essential amino acids (Neucere and Sumrell, 1979) and low protein digestibility, which is severely impaired by the cooking process (Axtell et al., 1981; Eggum, 1983; Mitaru, 1985; Oria et al., 1995).

Lactic acid fermentation is usually applied on the production of many sorghum porridges. This technology is able to improve nutritional and functional properties of sorghum (Belton and Taylor, 2004) as it increases starch digestibility and decreases resistant starch (El Tinay et al., 1979; Elkhalifa et al., 2006; Elkhalifa et al., 2004a; Elkhalifa et al., 2004b), provides a better essential amino acid composition (Au and Fields, 1981; Chavan, 1989; Chavan et al.,

1988; Kazanas and Fields, 1981; Sanni et al., 2001), and increases protein digestibility (Chavan et al., 1988; Hassan and El Tinay, 1995; Moneim et al., 1995).

Besides its rural utilization, sorghum is also consumed in some urban areas by cultural reasons. A study carried out with urban residents of Polokwane (the capital of the Limpopo province in South Africa) reveals that sorghum, which is easily purchasable in town, is widely consumed mainly as soft porridge, but also as thick porridge, fermented porridge and sorghum beer (Bichard et al., 2005). Fermentation is, however, a time consuming technology that is not suitable to the urban populations. In the same study, interviewed people complained that sorghum porridge takes too long to prepare for weekday breakfasts and a consumer demand for sorghum-based products and for its inclusion in infants' cereals used as weaning foods was reported. There is an unsatisfied demand for new sorghum-based products, which are convenient for breakfast (Bichard et al., 2005).

In this study, sorghum cooked flour was inoculated with pure cultures of lactic acid bacteria and protein quality, starch availability, viscosity and volatile organic compounds of the porridge were analyzed. The aim was to investigate the possibility of using commercial strains of lactic acid bacteria for the industrial scale production of sorghum fermented foods with improved nutritional qualities.

2. Materials and methods

2.1. Sorghum flour

Sorghum grains, of the Australian variety Jumbo, were purchased in a retail trade and ground with a coffee mill to pass through a 3×10^{-4} m sieve.

2.2. Bacterial strains and growth conditions

Lactobacillus brevis (DSM 6235), *Lactobacillus fermentum* (DSM 20052) and *Streptococcus thermophilus* (DSM 20617) were obtained from DMSZ (Braunschweig, Germany) in lyophilized form. All these species have been found in sorghum fermented products (Mugula et al., 2003a; Mugula et al., 2003b; Muyanja et al., 2003).

MRS broth (Merck - Darmstadt, Germany) was used for re-hydration. After re-hydration the bacteria were disseminated by streaking on MRS agar (Merck - Darmstadt, Germany) and incubated for 24 h at their optimal growth temperature (37 ºC for *L. fermentum* and *S. thermophilus* and 30 ºC for *L. brevis*).

2.3. Preparation of starter cultures

Aqueous suspensions of starter cultures were prepared from 24 h cultures of each one of the LAB species on agar plates. With sterile loops, LAB cultures were transferred to physiological serum (NaCl 0.9% w/v) and stirred in a vortex. Dilutions were made in order to obtain inoculums containing about 10^7 CFU/mL, determined by optical densities. Optical densities at 600 nm (OD_{600}) were measured using a Shimadzu UV-160A spectrophotometer (Tokyo, Japan). Cellular concentrations of each inoculum were obtained from calibration curves between OD_{600} and the number of colonies/mL determined by standard plate count.

2.4. Flour fermentation

For lactic fermentation, a sample of sorghum flour (15 g) was mixed with sterilized water (1:10 w/v), in sealed E-flasks. This mixture was boiled, for starch gelatinization, during 1 minute under vigorous stirring prior autoclaving at 121 ºC for 15 min. After cooled, at room temperature (*ca.* 25ºC), this sample was inoculated with 1.667 x 10^{-3} L of each bacterial

suspension of *Lactobacillus brevis, Lactobacillus fermentum* and *Streptococcus thermophilus*. The sample was incubated for five days at 30ºC.

An unfermented control sample was prepared as described before omitting the inoculation step. To keep the same conditions of fermented samples, 5 x 10^{-3} L of physiological serum (NaCl 0.9% w/v) was added.

2.5. Samples Preparation

After fermentations were completed, pH of samples was measured and each of the samples was divided in two porridge portions. The first one was used to determine volatile organic compounds, total amino acids content, and apparent viscosity.

The second portion of each sample was centrifuged at 24,000*g* during 20 minutes (Sigma 3K30 centrifuge - Osterode am Harz, Germany) and the residues were freeze dried and ground again. These residues were used for the determination of total starch and amylose content, and for the *in vitro* digestibility assays for protein (IVPD) and starch (IVSD). The supernatants were used for the nuclear magnetic resonance (^{1}H NMR) analysis.

2.6. In vitro protein digestibility assay

Control and fermented samples were subjected to *in vitro* protein digestibility (IVPD) assay using pepsin (Sigma - P-7000 - 975 U/mg protein), as described by Nunes et al. (2004), with some modifications. Flour samples (1 x 10^{-1} kg in glass tubes) were stirred and digested with pepsin (20 mg pepsin/mL 0.1 M KH_2PO_4 pH 2 buffer) in a water bath (37^{o}C) for 0 (t0) and 120 minutes (t120). After this period of time, the digestions were stopped by the addition of 1 x 10^{-4} L of 2 M NaOH and each tube was placed in an ice bath. All samples were centrifuged (2500 x g, room temperature) for 3 minutes and the supernatants discarded. The residues were washed with 1 x 10^{-3} L of 0.1 M K_2HPO_4 pH 7 buffer, centrifuged and washed again with 1 x

10^{-3} L of water. These residues, with insoluble undigested proteins, were freeze-dried and weighed. The content of total N that remains in control samples (t0) and in samples after 120 minutes (t120) of protein digestion was determined by elementary analysis of N.

The proportion (as a function of initial total protein) of soluble proteic material prior to pepsin addition was calculated by the sum of soluble protein and amino acids values.

The percentage of insoluble proteins digested by pepsin was calculated by the difference between insoluble protein content at 0 (t0) and 120 (t120) minutes of digestion. The *in vitro* protein digestibility was defined as the sum of protein solubilized by endogenous and microbial enzymes and the proportion of insoluble protein digested by pepsin through the follow formula:

$$\text{IVPD} = \left(\frac{\text{SP} + \text{AA}}{\text{TP}} + \frac{\text{Protein content (t0)} - \text{Protein content (t120)}}{\text{Protein content (t0)}} \right) \text{x } 100$$

Where SP is the soluble proteins content prior pepsin digestion; AA is the free aminoacids content prior pepsin digestion; TP is the total protein; Protein content (t0) – Protein content (t120) is the content of proteins solubilized by pepsin in the IVPD assay and Protein content (t0) in the content of insoluble proteins prior IVPD assay

2.7. Total amino acids analysis

For the analysis of total amino acids, samples were submitted to acid hydrolysis with 6 M HCl during 24 h according to procedure described by Zumwalt (1987). The acid was removed by centrifugal evaporation at 40 ºC under vacuum (Univapo 100 H, Uniequip, Munich, Germany) and the residue was dissolved in 1 x 10^{-3} L of 0.1 M HCl. After filtration through a 45 x 10^{-6} m membrane, amino acids were derivatizated as described by MacKenzie (1987).

Amino acids were determined by GC-FID as heptafluorobutylisobutyl derivatives, using norleucine as internal standard. A Clarus 400 gas chromatography apparatus (Perkin Elmer) with a flame ionisation detector (FID) and equipped with a 30 m length DB-1 column (J&W Scientific, Folsom, CA, USA) with i.d 25 x 10^{-5} m and 1 x 10^{-7} m film thickness was used. The oven temperature program used was: initial temperature 70 ºC during 1 minute, a rise in temperature at a rate of 2 ºC min^{-1} until 170 ºC and then a rate of 16 ºC min^{-1} until 250 ºC and this temperature was maintained for 5 minutes. The injector and detector temperatures were, respectively, 250 and 260 ºC.

2.8. ^{1}H NMR

High resolution nuclear magnetic resonance (NMR) spectroscopy was used to evaluate the effects of fermentation on the chemical composition of the supernatants. The samples were prepared to contain 10% D_2O, used as the internal lock, and 0.02% sodium 3-(trimethylsilyl) propionate-d_4 (TSP-d_4), used as chemical shift and intensity reference, their pH adjusted to 4 and transferred to 5 x 10^{-3} m o.d. NMR tubes. The ^{1}H 1D NMR spectra were recorded at 27ºC on a Bruker Avance DRX-500 spectrometer, operating at 500.13 MHz for proton, using the 'noesypr1d' pulse sequence: RD–90º–t_1–90º–t_m–90º–acquire FID (Bruker library), where RD is the relaxation delay (5.0 s), t_1 represents the first increment in a NOESY experiment (3 μs), and t_m is the mixing period (100 ms). The water signal was suppressed by presaturation during the relaxation delay and mixing time. Each ^{1}H 1D spectrum consisted of 128 scans of 32K data points with a spectral width of 8012.82 Hz and an acquisition time of 2.04 s. Prior to Fourier transformation (FT), the free induction decays (FIDs) were zero-filled to 32k points and multiplied by an exponential line-broadening function of 0.3 Hz. The 1D spectra were

manually phased, baseline corrected and the chemical shifts referenced to the TSP resonance to 0 ppm.

2.9. Total starch determination

The amount of total starch was determined using a total starch determination kit (Megazyme International Ireland Limited- Wicklow, Ireland).

2.10. In vitro starch digestibility assay

In vitro starch digestibility of control and fermented sample were determined according to the method described by Dahlqvist (1964) with some modifications. Freeze-dried flour residues (100 mg in glass tubes) were suspended in 4 x 10^{-3} L of 0.05 M phosphate buffer, pH 6.5, and placed in a water bath at 76 ºC to promote starch gelatinization. After cooled, samples were incubated with 1.66 x 10^{-6} L of α-amylase (Sigma – A7720 - 870 U/mg protein) in a water bath at 37 ^{o}C for 60 minutes. After this period of time, the enzyme was inactivated by heating in a boiling water bath for 10 minutes. Simultaneously, control samples were carried out under the same conditions, inactivating α-amylase by heating at the moment of its addition. Samples were centrifuged (2500*g* at room temperature for 3 minutes). Supernatants were analyzed for released reducing sugars by the 3,5-dinitrosalicylic acid (DNS) colorimetric method, using glucose as standard (Miller, 1959).

The *in vitro* starch digestibility was defined as the percentage of sugars released by the action of amylase at 37^{o}C during 60 minutes. These values were calculated by difference between sugars released with and without the action of α-amylase.

2.11. Amylose content

For the measurement of amylose content, an amylose/amylopectin assay kit was used (Megazyme International Ireland Limited- Wicklow, Ireland).

2.12. Viscosimetric analysis

Shear rates developed with applied shear stresses for sorghum dispersions were measured a 20^{o}C using a controlled-stress rheometer (AR-1000, TA Instruments, New Castle, DE) fitted with a cone-plate geometry (diameter 40 mm and angle 4^{o}).

Apparent viscosity was compared at shear rates of 1.48 x 10^{2} s^{-1}.

2.13. Volatile Analysis

Volatile organic compounds were determined by headspace analysis using solid phase microextraction (HS-SPME). A SPME holder was used to perform headspace SPME manually. SPME holders for manual sampling and fibre used in the analyses were purchased from Supelco Inc. (Bellefonte, PA, USA). SPME device included a fused silica fibre, partially cross-linked with 75 x 10^{-6} m Carboxen/polydimethylsiloxane (CAR/PDMS), which was conditioned according to the manufacturer's recommendations (280 ºC for 90 min in the GC injector).

Sorghum porridges (0.008 kg of fermented and unfermented samples) were placed into an 18 x 10^{-3} L glass vial, which corresponds to a ratio of solid phase volume to headspace volume (1/β) of 1.0. The vials were capped with a cap containing a PTFE septum (Sigma-Aldrich Inc., Bellefonte, PA, USA) and placed in a thermostatted bath adjusted to 40.0 ± 0.1ºC to promote the transference of the compounds from the sample to the headspace. A 60 minutes time of partition between the samples and the headspace was used. After this step, the SPME fibre was manually inserted into the sample vial headspace and the compounds were extracted for 45 minutes. Blanks, corresponding to the analysis of the coating fibre not submitted to any extraction procedure were run between sets of three analyses. All measurements were made with three replicates, each one of a different aliquot.

2.13.1. Gas chromatography–quadrupole mass spectrometry (GC–qMS) analysis

The desorbed volatile compounds from SPME were separated and analyzed on a GC-qMS (Agilent Technologies 6890N Network gas chromatograph). The GC was equipped with a 30 m x 32 x 10^{-5} m (i.d.) DB-FFAP fused silica capillary column (J&W Scientific, Folsom, CA, USA), 25 x 10^{-8} m film thickness, connected to an Agilent 5973 mass selective detector. Injections were made in splitless mode (5 min) and the injector was at 250 ºC.

The injection port was lined with a 75 x 10^{-5} m i.d. splitless glass liner. The fibre, containing the headspace volatile compounds, was introduced into the injector for 15 min for desorption of the compounds.

The oven temperature was programmed from 35 to 220 ºC at 2 ºC/min rate and the transfer line was heated at 250 ºC. The helium carrier gas had a column head pressure of 12 psi. The mass spectrometer was operated in the electron impact mode at 70 eV, scanning the range m/*z* 30–300 in a 1 s cycle, in a full scan mode acquisition. Identification of volatile compounds was achieved by comparison with the library data system of the GC–qMS equipment (Wiley 275).

2.14. Statistical analysis

All values are expressed as means and standard deviation for three replicates. Mean values of treatments were compared by Student's t test. Differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Protein quality

Protein quality of sorghum fermented with *Lactobacillus brevis, Lactobacillus fermentum* and *Streptococcus thermophilus* was evaluated in this work by *in vitro* protein digestibility and amino acids composition of the porridge.

The i*n vitro* protein digestibility (IVPD) increased from 19.66 (±0.49) to 64.75 % (±0.51) with fermentation. The increase of sorghum digestibility was also found in studies with sorghum porridges fermented with endogenous flora (Graham et al., 1986; Mertz et al., 1984; Taylor and Taylor, 2002).

Besides the increase on protein digestibility, a better amino acids balance was observed on fermented sorghum, with an increase in the essential amino acids valine, isoleucine, and methionine (Table 1). An increase in non-essential amino acids alanine, aspartic acid, glutamic acid, and tyrosine was also promoted by fermentation. This modification on amino acid composition is a result of a *de novo* production of amino acids from metabolic intermediates during the growth cycle of bacteria. The increase in valine, isoleucina, methionine, and tyrosine was found on previous studies on fermented sorghum porridges (Au and Fields, 1981; Kazanas and Fields, 1981) and *kisra* bread (El Tinay et al., 1979).

The NMR spectra of soluble components of the samples (Figure 1) revealed an increase of free amino acids phenylalanine, tyrosine, γ-amino-butyric acid (GABA), alanina and valine when comparing the fermented sample (Figure 1b) with the control sample (Figure 1a).

Table 1 – Amino acids analysis of control sample and sample fermented with *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*.

Amino acids	***Control Sample (%)*** [1]	***Fermented Sample (%)*** [1]
Alanine	8.6 ± 0.3	10.1 ± 0.3 [2]
Glycine	3.3 ± 0.1	3.4 ± 0.0
Valine	9.0 ± 0. 2	10.5 ± 0.2 [2]
Threonine	1.9 ± 0. 1	1.7 ± 0.1
Serine	6.3 ± 0.1	6.2 ± 0.1
Leucine	25.8 ± 0.1	19.0 ± 0.2 [2]
Isoleucine	1.9 ± 0.1	2.1 ± 0.1 [2]
Proline	8.5 ± 0.3	8.3 ± 0.2
Methionine	0. 7 ± 0.1	1. 1 ± 0.1 [2]
Aspartic acid	6.9 ± 0.2	8.2 ± 0.2 [2]
Phenylalanine	4.0 ± 0.2	4.2 ± 0.2
Glutamic acid	14.8 ± 0.2	17.6 ± 0.2 [2]
Lysine	1.3 ± 0.1	1.3 ± 0.2
Tyrosine	2.6 ± 0.1	2.9 ± 0.2 [2]
Arginine	4.1 ± 0.2	3. 5 ± 0.2 [2]

[1] Mean of three replicates ± standard deviation.
[2] Differences from control sample were considered significant at $p < 0.05$.

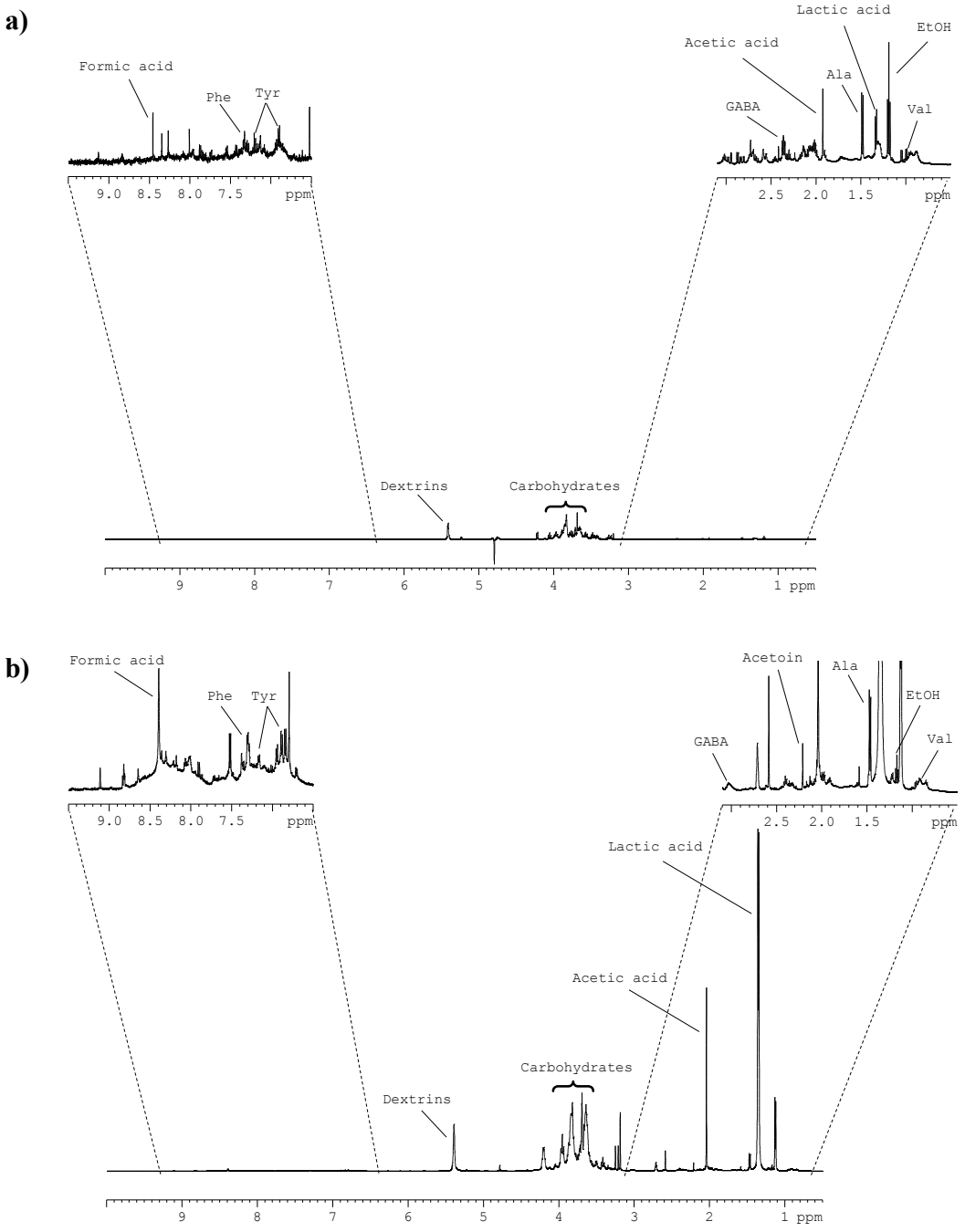

Figure 1 – **-** ^{1}H NMR spectra of **(a)** control sample and **(b)** sorghum fermented with *Lactobacillus brevis*, *Lactobacillus fermentum* and *Streptococcus thermophilus*.

3.2. Starch availability

Differences between starch content of control (64.03 ± 2.97) and fermented sample (65.88 ± 0.88) are not significant ($p < 0.05$). However, this could be a result of starch concentration in fermented samples as a consequence of loss of dry matter of the insoluble residue due to the consumption of non starch components by the microorganisms. The same effect of starch concentration was found in a previous work (Correia et al., 2004). In fact, the decrease of starch in fermented sample is confirmed by the NMR spectra of the soluble fraction of this sample, which shows an increase of a signal at 5.4 (ppm), arising from the H1 protons from the glucose anomeric carbon involved in $\alpha(1\rightarrow4)$ glycosidic linkages of dextrins and of signals from dextrins between 3.2 and 4.2 (ppm) (Figure 1). The occurrence of these signals in control sample (Figure 1a) is a consequence of starch gelatinization by cooking. With fermentation, starch was hydrolyzed by microbial amylases, which promoted the solubilisation of dextrins to the liquid phase (Figure 1b).

The *in vitro* starch digestibility (IVSD) suffered an increase from 33.8 (± 0.2) to 50.3 % (± 0.2) with fermentation (Table 1). Similar values were obtained in other studies with sorghum dough (Elkhalifa et al., 2004a; Hassan and El Tinay, 1995; Kazanas and Fields, 1981), indicating that the inoculum used in this work and the endogenous microflora that acts on traditional fermented porridges have similar abilities to increase starch availability.

Some authors suggested that starch granules are completely enclosed in a very compact protein matrix, in such way that endosperm proteins restrict starch granules from fully gelatinization (Chandrashekar and Kirleis, 1988; Elkhalifa et al., 2006; Elkhalifa et al., 2004a). According to Elkhalifa et al. (2006; 2004a), the increase in IVSD with fermentation is related

with changes in endosperm protein fraction that makes starch more accessible to the digestive enzymes. These authors suggest that changes promoted by microbial proteases on protein coating structure leads to the release of the previously tightly packed starch granules. In fact electrophorogrammes from fermented samples show less high molecular weight aggregates (results not shown).

Starch digestibility is also affected by the amylose and amylopectin content. Starch digestibility in low-amylose sorghum was reported to be higher than in normal sorghum, corn and pearl millet grains (Hibberd et al., 1982). Results of amylose/amylopectin assay revealed that amylose content decreased from 50.8 (± 0.3) to 31.3 % (± 0.2) with fermentation. The observed decrease in amylose content could be another explanation for the superior IVSD of the fermented sample. Amylose reduction is a consequence of starch hydrolysis by bacterial amylases. The assumption of starch hydrolysis is confirmed.

3.3. Viscosimetric analysis

There has been little work specifically addressing the effect of fermentation on viscosity and the findings are highly contradictory. Viscosity appears to vary according to the method of fermentation and particularly to the microorganism responsible (Wambugu et al., 2003).

In this work, the apparent viscosity at a shear rate of $1.48 \times 10^{2}\ s^{-1}$ was 1.52×10^{-1} Pa.s for the unfermented sample whereas a value of only 2.97×10^{-2} Pa.s was obtained for fermented sample at the same conditions. This decrease of 80.46 % in apparent viscosity with fermentation is a result of bacterial amylases and proteases of the selected starter cultures that hydrolyzed starch and proteins, thus decreasing the bulk caused by starch gelatinization. We can conclude that with the fermentation conditions applied in this work it is possible to reduce

porridge viscosity, making thinner sorghum porridge, without lowering its nutrient and energy density.

3.3. Volatile Analysis

During cereal fermentations several volatile compounds are formed, which contribute to a complex blend of flavors in the products (Blandino et al., 2003). The proteolytic activity of bacteria may produce precursors of flavor compounds, such as amino acids, which could be deaminated or decarboxilated to organic acids and aldehydes. On the other hand, these aldehydes could be oxidized to acids or reduced to alcohols (Erbas et al., 2005; Mugula et al., 2003b)

Volatile organic compounds (VOC) found by HS-SPME analysis in the fermented porridge include organic acids (butyric acid, acetic acid and isobutyric acid), alcohols (1-butanol, 2-butanol and ethanol), esters (ethyl butanoate) and ketones (2-butanone). Butyric acid was the major VOC found on the fermented porridge. Acetic acid and ethanol were also determined by ^{1}H NMR analysis of the porridge supernatant, in addition to formic acid, lactic acid and acetoin (Figure 1b).

The organic acids formed are responsible for the observed decrease in pH from 6.41 to 4.29 with fermentation.

Lactic and acetic acids have been reported to be flavor enhancers and are responsible for the sour taste of fermented porridges (Onyango et al., 2000). Acetoin are associated to the butter aroma (Muyanja et al., 2003)

The majority of the found compounds were already reported in fermented sorghum porridges. Characterization of organic compounds of *Ogi* (a Nigerian fermented porridge) reveals the presence of lactic, acetic and butyric acids (Banigi and Muller, 1972; Osungbaro,

2009), all contributing to the flavor of *Ogi*. Lactic acid, acetic acid, 1-butanol, ethyl butanoate and ethanol were found in *Uji*, another type of thin porridge (Masha et al., 1998). Acetoin, 2-butanone and lactic, acetic and formic acids were also found in *Togwa* (Mugula et al., 2003a; Mugula et al., 2003b).

NMR shows that control sample also presents very low concentration of ethanol, acetic acid formic acid and lactic acid (Figure 1a). The presence of ethanol, acetic acid and lactic acid prior fermentation was previously reported (Masha et al., 1998; Onyango et al., 2004a).

4. Conclusions

The selection of pure cultures to be used as starters for sorghum fermentation is important in the development of production of weaning foods with superior nutritional quality.

Bacterial strains used in this work were able to modify grain constituents rendering proteins and starch more digestible and to enhance protein quality by the increase in essential amino acids. Additionally, bacterial enzymes of *Lactobacillus brevis* (DSM 6235), *Lactobacillus fermentum* (DSM 20052) and *Streptococcus thermophilus* (DSM 20617) were able to reduce viscosity making thinner sorghum porridge with the same nutritional density. This is important as the bulk caused by starch gelatinization is one of the major problems to be solved for the preparation of affordable fermented weaning foods ((Nout and Ngoddy, 1997).

Organoleptic characteristics are important for the consumers' acceptation of sorghum products. Volatile organic compounds formed during cereal fermentations play an essential contribution to the formation of product flavors. The sorghum porridge obtained in this work presents several organic compounds usually found on traditional porridges. However, a further sensory analysis of this product must be done.

We conclude that a mixed inoculum composed of *Lactobacillus brevis* (DSM 6235), *Lactobacillus fermentum* (DSM 20052) and *Streptococcus thermophilus* (DSM 20617) could be a promising starter for the production of commercial weaning foods with the requisites required by African consumers.

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CAPÍTULO 6 – CONCLUSÕES

O estudo dos efeitos produzidos pelas diferentes formas de processamento permitiu concluir que não existe uma relação directa entre a extractibilidade das proteínas e a digestibilidade das mesmas. A pipocagem, tal como o cozimento, promoveu uma diminuição na extractibilidade das proteínas de sorgo, no entanto, não promoveu alterações no valor da digestibilidade proteica. Verificou-se, ainda, que a água exerce um papel fundamental na diminuição da digestibilidade proteica com o aquecimento, tendo-se observado alterações muito ténues na digestibilidade quando se promoveu o aquecimento em banho-maria (aquecimento a seco). Por outro lado, a análise das amostras submetidas ao tratamento por alta-pressão mostrou que o amido e os lípidos apresentam um papel preponderante na digestibilidade. Estes resultados mostram que, para além da formação de pontes dissulfeto, interacções proteínas-amido-lípidos, com possível formação de complexos, poderão estar por trás da diminuição da digestibilidade com o cozimento.

Da análise dos efeitos promovidos pela alta-pressão, concluiu-se que a aplicação desta tecnologia como tratamento prévio ao cozimento permite um aumento da digestibilidade do sorgo cozido para valores próximos aos do sorgo cru.

A fermentação e a germinação são duas formas de processamento que permitem reverter os efeitos adversos do cozimento devido à acção de amilases, proteases e lipases microbianas e endógenas, respectivamente.

A simulação do processo tradicional de fermentação do sorgo conduziu a resultados semelhantes aos mencionados na literatura para fermentações espontâneas. Deste modo, os resultados obtidos com a fermentação tradicional puderam servir de comparação com os obtidos com as espécies comerciais puras utilizadas neste trabalho.

Foi possível verificar que a fermentação da farinha de sorgo utilizando como inóculo as estirpes bacterianas das espécies *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus* promoveu, comparativamente à fermentação tradicional, um aumento mais acentuado na digestibilidade proteica. Este facto deveu-se à conjugação da

elevada capacidade proteolítica das espécies *Lactobacillus fermentum* e *Streptococcus thermophilus* com a elevada capacidade amilolítica da espécie *Lactobacillus brevis*. Conseguiu-se assim obter melhorias na qualidade do produto final, utilizando um inóculo mais simples e seguro.

A utilização das referidas espécies resultou num ataque às proteínas e ao amido e, consequentemente, no incremento da digestibilidade destes dois biopolímeros, na diminuição na viscosidade do produto final e numa melhoria no balanço em aminoácidos essenciais. Estes aspectos são muito importantes em termos nutricionais, sobretudo quando se considera o consumo de sorgo como alimento complemantar ao desmame. Verificou-se ainda ocorrência de vários compostos orgânicos usualmente encontrados em produtos tradicionais de sorgo.

A análise electroforética revelou que, como consequência do ataque proteolítico promovido pela fermentação com as referidas bactérias lácticas, ocorreu uma diminuição mais acentuada no teor de aglomerados proteicos de elevado peso molecular, monómeros α, β e γ e oligómeros de 45 kDa e 66 kDa. No caso do monómero β, o teor diminuiu para valores inferiores aos detectáveis pelo método. O oligómero de 45 kDa, formado pelo aquecimento e não redutível na presença de redutores fortes como o 2-mercaptoetanol, também não desapareceu com a fermentação. Contudo, esta proteína sofreu uma maior diminuição com este inóculo. Este facto poderá estar relacionado com a digestibilidade proteica superior desta amostra.

A conjugação de leveduras com as bactérias lácticas seleccionadas não se mostrou vantajosa, tendo resultado na diminuição da actividade proteolítica do inóculo e, consequente, na diminuição da digestibilidade proteica.

A incubação da farinha com malte previamente à fermentação promoveu por si só os mesmos efeitos que a fermentação, o que poderia levar a conclusão de que seria uma alternativa, mais económica e acessível ao nível caseiro, à preparação de papas de sorgo. Contudo, o facto das enzimas endógenas do grão promoverem um ataque tão extenso ao amido reflecte-se numa enorme desvantagem em termos nutricionais. A clivagem do amido em açúcares de mais rápida utilização por parte do organismo promove um aumento do índice glicémico que se traduz em inconvenientes para a saúde humana.

Os resultados obtidos na fermentação com a microflora presente nos grãos de Kefir também foram bastante satisfatórios em termos nutricionais. Dada a relativa facilidade de obtenção dos grãos de Kefir, este inóculo pode representar uma alternativa para a realização de fermentação ao nível doméstico.

Numa análise mais geral do trabalho, podemos concluir que a fermentação da farinha de sorgo com *Lactobacillus brevis*, *Lactobacillus fermentum* e *Streptococcus thermophilus* é uma alternativa bastante viável na preparação de alimentos a base de sorgo, com vantagens, comparativamente à fermentação tradicional, no que respeita qualidade nutricional do produto final.

Os resultados obtidos apontam para a possibilidade de utilização das espécies espécies *L. brevis*, *L. fermentum* e *S. thermophilus* como inóculo para uma farinha de sorgo previamente cozida, da qual pode resultar um produto alimentar de fácil preparação, seguro em termos alimentares e enriquecido em termos nutricionais. O tipo de fermentação sugerido pode ser utilizado a nível industrial como forma de processamento que aumenta o tempo de preservação do sorgo, incrementa as suas características biológicas e permite uma diminuição no tempo de preparação, indo assim ao encontro das necessidades dos consumidores das zonas urbanas.

Como trabalho futuro, sugere-se um estudo mais aprofundado acerca das características organolépticas do produto resultante, como forma de medição da aceitação dos consumidores, por exemplo, através da formação de um painel de provadores.

Num trabalho futuro, será também importante avaliar as vantagens da utilização da tecnologia de alta-pressão previamente à fermentação. Para além das referidas vantagens da alta-pressão como tecnologia que permite reverter os efeitos deletérios do cozimento, a sua utilização poderia simultaneamente funcionar como forma de esterilização da farinha previamente à sua inoculação com as bactérias comerciais seleccionadas.

Em termos microbiológicos, seria ainda importante a monitorização das estipes predominantes, bem como dos efeitos promovidos pelas mesmas, em cada estágio da fermentação.

O facto da utilização dos inóculos compostos por bactérias lácticas e leveduras não ter resultado numa simbiose favorável entre espécies, poderá ser consequência de não se ter promovido uma maior adaptação entre bactérias e leveduras através de propagações contínuas. Será assim importante ter este aspecto em conta na continuação deste trabalho.

CAPÍTULO 7 - REFERÊNCIAS BIBLIOGRÁFICAS

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